

OPEN ACCESS



African Journal of
Microbiology Research

28 May 2018
ISSN 1996-0808
DOI: 10.5897/AJMR
www.academicjournals.org



**ACADEMIC
JOURNALS**
expand your knowledge

ABOUT AJMR

The African Journal of Microbiology Research (AJMR) is published weekly (one volume per year) by Academic Journals.

The African Journal of Microbiology Research (AJMR) provides rapid publication (weekly) of articles in all areas of Microbiology such as: Environmental Microbiology, Clinical Microbiology, Immunology, Virology, Bacteriology, Phycology, Mycology and Parasitology, Protozoology, Microbial Ecology, Probiotics and Prebiotics, Molecular Microbiology, Biotechnology, Food Microbiology, Industrial Microbiology, Cell Physiology, Environmental Biotechnology, Genetics, Enzymology, Molecular and Cellular Biology, Plant Pathology, Entomology, Biomedical Sciences, Botany and Plant Sciences, Soil and Environmental Sciences, Zoology, Endocrinology, Toxicology. The Journal welcomes the submission of manuscripts that meet the general criteria of significance and scientific excellence. Papers will be published shortly after acceptance. All articles are peer-reviewed.

Contact Us

Editorial Office: ajmr@academicjournals.org

Help Desk: helpdesk@academicjournals.org

Website: <http://www.academicjournals.org/journal/AJMR>

Submit manuscript online <http://ms.academicjournals.me/>

Editors

Prof. Adriano Gomes da Cruz
*University of Campinas (UNICAMP),
Brazil.*

Prof. Ashok Kumar
*School of Biotechnology
Banaras Hindu University Uttar Pradesh,
India.*

Dr. Mohd Fuat Abd Razak
*Infectious Disease Research Centre,
Institute for Medical Research, Jalan
Pahang, Malaysia.*

Dr. Adibe Maxwell Ogochukwu
*Department of Clinical Pharmacy and
Pharmacy Management,
University of Nigeria
Nsukka, Nigeria.*

Dr. Mehdi Azami
*Parasitology & Mycology Department
Baghaeei Lab.
Isfahan, Iran.*

Dr. Franco Mutinelli
*Istituto Zooprofilattico Sperimentale delle
Venezie Italy.*

Prof. Ebiamadon Andi Brisibe
*University of Calabar,
Calabar,
Nigeria.*

Prof. Nazime Mercan Dogan
*Department of Biology
Faculty of Science and Arts
University Denizli Turkey.*

Prof. Long-Liu Lin
*Department of Applied Chemistry
National Chiayi University
Chiayi County Taiwan.*

Prof. Natasha Potgieter
*University of Venda
South Africa.*

Dr. Tamer Edirne
*Department of Family Medicine
University of Pamukkale
Turkey.*

Dr Kwabena Ofori-Kwakye
*Department of Pharmaceutics
Kwame Nkrumah University of Science &
Technology
Kumasi, Ghana.*

Dr. Tülin Askun
*Department of Biology
Faculty of Sciences & Arts
Balikesir University Turkey.*

Dr. Mahmoud A. M. Mohammed
*Department of Food Hygiene and Control
Faculty of Veterinary Medicine
Mansoura University Egypt.*

Editors

Dr. James Stefan Rokem
Department of Microbiology & Molecular Genetics
Institute of Medical Research Israel – Canada
The Hebrew University – Hadassah Medical School Jerusalem, Israel.

Dr. Afework Kassu
University of Gondar
Ethiopia.

Dr. Wael Elnaggar
Faculty of Pharmacy
Northern Border University
Rafha Saudi Arabia.

Dr. Maulin Shah
Industrial Waste Water Research Laboratory
Division of Applied & Environmental Microbiology, Enviro Technology Limited
Gujarat, India.

Dr. Ahmed Mohammed
Pathological Analysis Department
Thi-Qar University College of Science
Iraq.

Prof. Naziha Hassanein
Department of Microbiology
Faculty of Science
Ain Shams University
Egypt.

Dr. Shikha Thakur
Department of Microbiology
Sai Institute of Paramedical and Allied Sciences India.

Dr. Samuel K Ameyaw
Civista Medical Center
USA.

Dr. Anubrata Ghosal
Department of Biology
MIT - Massachusetts Institute of Technology
USA.

Dr. Bellamkonda Ramesh
Department of Food Technology
Vikrama Simhapuri University
India.

Dr. Sabiha Yusuf Essack
Department of Pharmaceutical Sciences
University of KwaZulu-Natal
South Africa.

Dr. Navneet Rai
Genome Center
University of California Davis USA.

Dr. Iheanyi Omezuruike Okonko
Department of Virology
Faculty of Basic Medical Sciences
University of Ibadan
Ibadan, Nigeria.

Dr. Mike Agenbag
Municipal Health Services,
Joe Gqabi,
South Africa.

Dr. Abdel-Hady El-Gilany
Department of Public Health & Community Medicine, Faculty of Medicine
Mansoura University
Egypt.

African Journal of Microbiology Research

Table of Content: Volume 12 Number 20 28 May, 2018

ARTICLES

- Antimicrobial activity of the extracts of *Albizia masikororum* R.Vig., a Fabaceae from Madagascar** 464
Anjarasoa Ravo Razafindrakoto, Lovarintsoa Judicael
Randriamampianina, Eliane Razafintsalama Vahinalahaja,
Hanitra Ranjana Randrianarivo, Danielle Aurore Doll Rakoto
and Victor Louis Jeannoda
- In-vitro antibiotic susceptibility profile of *Salmonella enterica* Serovar Typhi isolated from fecal specimens of humans in Umuahia metropolis, Abia State, Nigeria** 470
Emmanuel O. Ekundayo and Emmanuel Enya
- Isolation and identification of *Escherichia coli* and *Edwardsiella tarda* from fish harvested for human consumption from Zeway Lake, Ethiopia** 476
Adanech Beshada Haile and Temesgen Kassa Getahun

Full Length Research Paper

Antimicrobial activity of the extracts of *Albizia masikororum* R.Vig., a Fabaceae from Madagascar

Anjarasoa Ravo Razafindrakoto¹, Lovarintsoa Judicael Randriamampianina¹, Eliane Razafintsalama Vahinalahaja², Hanitra Ranjana Randrianarivo¹, Danielle Aurore Doll Rakoto¹ and Victor Louis Jeannoda^{1*}

¹Laboratory of Applied Biochemistry to Medical Sciences, Fundamental and Applied Biochemistry Department, Faculty of Sciences, University of Antananarivo, P. O. Box 906, Antananarivo 101, Madagascar.

²National Center for Application of Pharmaceutical Research (CNARP), P. O. Box 702, Antananarivo 101, Madagascar.

Received 6 April, 2018; Accepted 21 May, 2018

This study is aimed at the assessment of antimicrobial potential of *Albizia masikororum*, a Malagasy Fabaceae. Hexanic and methanolic extracts from fruit pods, stem bark, leaves and seeds were tested by disc diffusion and microdilution methods on 10 pathogenic microorganisms including four Gram positive bacteria, five Gram negative bacteria and one yeast. Only the leaf and seed methanolic extracts, LME and SME respectively, were active on some bacteria. LME and SME had a broad-spectrum activity, with SME more effective. The minimum inhibitory concentration (MIC) values of both extracts were <1000 µg/ml of which 30% <100 µg/ml, 20% between 100 and 500 µg/ml and 50% between 500 and 1000 µg/ml. SME MICs ranked from 6.10 µg/ml (*Streptococcus pneumoniae*) to 781.25 µg/ml (*Yersinia enterocolitica*) and those of LME from 97.65 µg/ml (*Streptococcus pneumoniae*) to 781.25 µg/ml (*Bacillus cereus*, *Streptococcus pneumoniae* and *Yersinia enterocolitica*). LME was bactericidal on all sensitive bacteria whereas SME was bactericidal on some and bacteriostatic on others. Both extracts contained different chemical groups known for their antimicrobial properties, saponins in SME and phenolic compounds in LME.

Key words: *Albizia masikororum*, antimicrobial activity, disc diffusion method, microdilution method, minimum inhibitory concentration, minimum bactericidal concentration.

INTRODUCTION

Globally, the number of multidrug-resistant strains has increased dramatically over the past 10 years (Roca et al., 2015; Sharifi-Rad, 2016) and new more resistant strains may be expected to emerge in the coming years (McGaw et al., 2008).

This situation may exhaust the chemical defenses at

our disposal. Therefore, research programs in alternative therapies including the search for new antimicrobial agents should be encouraged. Natural products still play a major role in the search for new medicines and plants represent a good source for new molecules of interest (Kuethe et al., 2009; Sharifi-Rad et al., 2017a, b, c, d, e;

*Corresponding author. E-mail: victor_jeannoda@yahoo.fr.

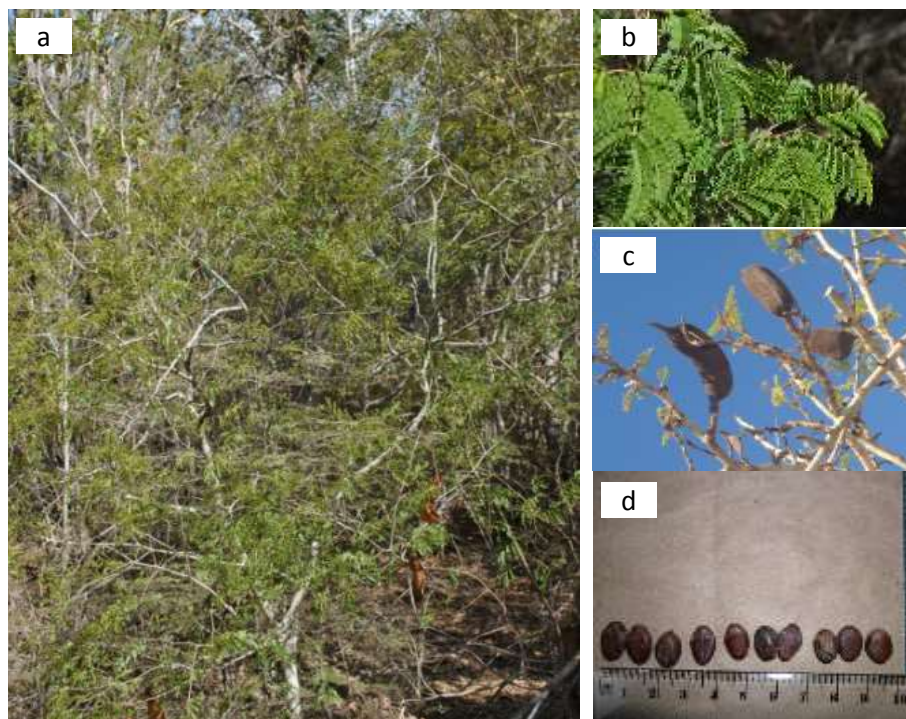


Figure 1. *Albizia masikororum*: (a) the whole plant; (b) leaves; (c) fruits, and (d) seeds. Source: The authors and Silo National des Graines Forestières (SNGF).

Salehi et al., 2017).

Malagasy plants are good candidates in view of the diversity of Malagasy flora whose originality could promise original molecules.

The genus *Albizia* is a group of plants that includes about 150 species and occurs in tropical regions. Worldwide, it has many uses including control of pests such as pathogenic microorganisms (Agyare et al., 2006; Sarkiyayi et al., 2011; Odeyemi et al., 2014). The Malagasy species studied also showed *in vitro* antimicrobial properties (Randriamampianina et al., 2017). In this work, the antimicrobial potential of the endemic species *A. masikororum*, which has already shown appreciable biological properties on mice and chicken (Randrianarivo et al., 2014) was studied.

MATERIALS AND METHODS

Plant materials

A. masikororum is a large shrub or tree up to 25 m tall (Figure 1) growing throughout the western part of Madagascar, from the North to the South. It was collected in Belo-sur-Tsiribihina/Betioky (West of Madagascar). Voucher specimens of the plant were deposited in the herbarium of Silo National des Graines Forestières (SNGF) under the reference number SNGF 873.

Microorganism strains

The 10 microorganisms used in this study included 4 Gram (+)

bacteria, 5 Gram (-) bacteria and 1 yeast (Table 1). They were maintained on agar slants at 4°C and cultured on a fresh appropriate agar plates 24 h prior to any antimicrobial test.

Chemicals for antimicrobial assay

Neomycin (30 µg/ml/disc) (Bio-Rad F-92430, Marnes-la Coquette, France) were used as antibiotic references. Miconazole (500 µg) (Bio-Rad F 92430 Marnes-la-Coquette, France) was used as antifungal reference for yeasts.

Preparation of hexane and methanolic extracts

Ground seeds, leaves, pods and stem bark powder (50 g) were delipidated with hexane (3 X 250 ml), then extracted with methanol (3 X 250 ml). After filtration using a Whatman filter paper, extracts were evaporated to dryness under reduced pressure.

Phytochemical screening

The reactions of chemical group detection were those developed by Marini-Bettolo et al. (1981).

Antimicrobial assays

All the materials and methods used for antimicrobial assay were detailed in a previous study (Randriamampianina et al., 2017).

The results were interpreted using the scale of Ponce et al. (2003) and Celikel et al. (2008). With the disc diffusion method, bacteria are not considered sensitive for an inhibition zone diameter (IZD) less than 8 mm, sensitive for IZD of 9 to 14 mm, very

Table 1. List of microorganisms used.

Strain	Reference	Gram
<i>Staphylococcus aureus</i>	ATCC 25923	+
<i>Bacillus cereus</i>	ATCC 14579	+
<i>Streptococcus pneumoniae</i>	ATCC 6305	+
<i>Streptococcus pyogenes</i>	ATCC 19615	+
<i>Enterobacter aerogenes</i>	ATCC 13048	-
<i>Enterobacter cloacae</i>	ATCC 13047	-
<i>Yersinia enterocolitica</i>	ATCC 23715	-
<i>Pseudomonas aeruginosa</i>	ATCC 10145	-
<i>Clostridium perfringens</i>	ATCC 13124	-
<i>Candida albicans</i>	ATCC 10231	-

Table 2. Extraction yields of the different crude extracts.

Organ	Pod		Bark		Leaf		Seed	
Extraction solvent	Hexane	Methanol	Hexane	Methanol	Hexane	Methanol	Hexane	Methanol
Designation	PHE	PME	BHE	BME	LHE	LME	SHE	SME
Yield (% w/w)	2.01	4.61	0.95	2.38	1.17	4.74	3.36	19.01

sensitive for IZD of 15 to 19 mm and extremely sensitive for IZD larger than 20 mm.

For each extract, the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined on susceptible strains only. The standards used to interpret MIC results were those of Dalmarco et al. (2010). For crude extracts and fractions, a MIC lower than 100 µg/mL was considered as an excellent effect, from 100 to 500 µg/ml as moderate, from 500 to 1000 µg/mL as weak, and over 1000 µg/ml as inactive.

The extract action is bactericidal or fungicidal when its ratio MBC/MIC or MFC/MIC is ≤ 4 and bacteriostatic and fungistatic when the ratio is >4 (Djeussi et al., 2013; Bouharb et al., 2014; Chamandi et al., 2015).

RESULTS

Extraction yields

The different crude extracts were obtained with yields ranging from 0.95% for BHE to 19.01% for SME (Table 2).

Phytochemical analysis

The major secondary metabolites identified in seed methanolic extract (SME) and leaf methanolic extract (LME) are shown in Table 3. In SME, saponins, unsaturated sterols, triterpenes and cardenolides were present, while alkaloids, tannins, flavonoids, leucoanthocyanins, coumarins, iridoids, steroids, and anthraquinones were not detected. LME contained tannins, flavonoids, leucoanthocyanins and triterpenes, while alkaloids, saponins, unsaturated sterols, cardenolides, steroids, coumarins, iridoids, steroids and anthraquinones were absent.

Antimicrobial activity

Disc diffusion

The effects of the various extracts of seeds, pods, bark and leaves at a concentration of 1 mg/disc on the microorganisms are presented in Table 4.

LME and SME only displayed activity on some bacteria. Their IZD values ranked from 11.5 to 16.5 mm for LME and from 9 to 15 mm for SME. They were less effective than neomycin which is a pure product. None of the extracts was active against *Pseudomonas aeruginosa* and *Candida albicans*.

MIC and MBC values

MIC and MBC values were determined for LME and SME only for active extracts on sensitive bacteria by the disc diffusion method (Table 5).

All the MIC values were <1000 µg/ml: 27.2% were <100 µg/ml, 27.2% between 100 and 500 µg/ml and 45.6% between 500 and 1000 µg/ml. There were more strains sensitive to SME (7 strains) than to LME (4 strains). SME MICs ranked from 6.10 µg/ml (*Streptococcus pneumoniae*) to 781.25 µg/ml (*Yersinia enterocolitica*) and those of LME from 97.65 µg/ml (*Streptococcus pneumoniae*) to 781.25 µg/ml (*Bacillus cereus*, *Streptococcus pneumoniae* and *Yersinia enterocolitica*).

The best activities were observed with SME against *Streptococcus pneumoniae* (6.10 µg/ml) and *Streptococcus pyogenes* (24.41 µg/ml).

LME exerted a bactericidal effect against all bacteria

Table 3. Phytochemical screening of seed powder and organ methanolic extracts.

Chemical group	Test	Seed powder	Methanolic extracts from				
			Seeds	Leaves	Pods	Bark	
Alkaloids	Mayer	-	-	-	-	-	
	Wagner	-	-	-	-	-	
	Dragendorff	-	-	-	-	-	
Tannins	Gelatin 1%	+	-	+	-	+	
	Salted gelatin	+	-	+	-	-	
	FeCl ₃	+	-	+	-	-	
Phenolic compounds	Flavonoides	Flavonols	-	-	+	+	+
		Flavones	-	-	-	-	-
		Flavonones	-	-	-	-	-
	Leucoanthocyanins	-	-	+	+	+	
	Anthraquinones	-	-	nd	nd	-	
	Coumarins	nd	-	nd	nd	-	
Terpenoids	Triterpenes	Liebermann-Burchard	+	+	+	-	-
		Steroids	-	-	-	-	-
	Unsaturated sterols	Salkowski	+	+	-	-	-
	Iridoids	-	nd	nd	-	nd	
Heterosides	Saponins	+	+	-	-	-	
	Cardenolides	nd	+	nd	nd	nd	
	Cyanogenic glycosides	nd	-	nd	nd	nd	

+: Positive test; -: Negative test; nd: Not determined.

Table 4. Effects (IZD in mm) of *A. masikororum* extracts by disc diffusion method at a concentration of 1 mg/disc.

Strain	PHE	PME	BHE	BME	LHE	LME	SHE	SME	Neomycin (30 µg/disc)	Miconazole (500 µg/ml)	
Gram (+) bacteria	<i>Bacillus cereus</i>	6	6	6	6	6	11.5	6	6	21	-
	<i>Staphylococcus aureus</i>	6	6	6	6	6	13	6	10	18	-
	<i>Streptococcus pneumoniae</i>	6	6	6	6	6	16.5	6	9	23	-
	<i>Streptococcus pyogenes</i>	6	6	6	6	6	7.5	6	15	24	-
Gram (-) bacteria	<i>Clostridium perfringens</i>	6	6	6	6	6	7	6	13	22	-
	<i>Enterobacter aerogenes</i>	6	6	6	6	6	6	6	8	19	-
	<i>Enterobacter cloacae</i>	6	6	6	6	6	7	6	12	27	-
	<i>Pseudomonas aeruginosa</i>	6	6	6	6	6	6	6	6	18	-
	<i>Yersinia enterocolitica</i>	6	6	6	6	6	14.5	6	9	20	-
Yeast	<i>Candida albicans</i>	6	6	6	6	6	6	6	nd	-	25

nd: Not determined.

sensitive to it whereas SME had a bactericidal effect on 4 bacteria and bacteriostatic effect on 3 others.

DISCUSSION

Of all the 8 extracts tested, only methanolic extracts from leaves (LME) and seeds (SME) displayed antimicrobial

activity.

The antibacterial compounds in the two extracts did not belong to the same chemical groups: Phenolic compounds for LME and saponins for SME. As these extracts were only semi-purified, the study results did not yet allow the conclusion on the number of active compounds in each one of them.

Table 5. MIC, MBC, MFC ($\mu\text{g/ml}$) and MBC/MIC or MFC/MIC of *A. masikororum* seed extracts.

Strain	Extract	MIC	MBC or MFC	MBC/MIC or MFC/MIC	
Gram (+) Bacteria	<i>Bacillus cereus</i>	LME	781.25	781.25	1
	<i>Staphylococcus aureus</i>	SME	195.31	>1000	>4
		LME	781.25	>1000	2
	<i>Streptococcus pneumoniae</i>	LME	97.65	97.65	1
		SME	6.10	24.41	4
	<i>Streptococcus pyogenes</i>	SME	24.41	48.82	2
Gram (-) Bacteria	<i>Clostridium perfringens</i>	SME	195.31	>1000	>4
	<i>Enterobacter aerogenes</i>	SME	195.31	>1000	>4
	<i>Enterobacter cloacae</i>	SME	781.25	>1000	>4
	<i>Yersinia enterocolitica</i>	SME	781.25	781.25	1
		LME	781.25	>1000	4

LME was active against 4 and SME against 7 of the 10 strains tested but unlike other Malagasy *Albizia* species so far studied (Rakoto et al., 2011; Randriamampianina et al., 2017), both extracts were not active against *Candida albicans*. Generally, the strain sensitivity varied significantly with the extract. For example, *Clostridium perfringens* and *Enterobacter cloacae* were sensitive to SME but resistant to LME whereas *Bacillus cereus* was sensitive to LME but not to SME. This difference could lie in the fact that the two extracts contained very different chemical groups. SME was rich in saponins and LME in phenolic compounds. Both chemical groups are known for their antimicrobial properties.

LME and SME displayed an excellent effect against *Streptococcus pneumoniae* and *Streptococcus pyogenes*. Those two bacteria, because of their high prevalence in infectious diseases and their resistance to various antibiotics, have become a serious clinical and health problem in many countries (Nueremberger and Bishai, 2004; Ardanuy et al., 2014; Lu et al., 2017; Sekizuka et al., 2017; Zhao et al., 2017).

In comparison with antimicrobial activity of other malagasy *Albizia* assessed in similar conditions (Randriamampianina et al., 2017), LME and SME gave MIC values much lower than 1000 $\mu\text{g/ml}$, and therefore far more effective compared to the seed extracts from *A. aurisparsa* against *B. cereus* (MIC = 1980 $\mu\text{g/ml}$) and from *A. mahalao* (MIC = 3750 $\mu\text{g/ml}$) and *A. polyphylla* (MIC = 2420 $\mu\text{g/ml}$) against *Staphylococcus aureus*. However, their activities were more or less similar to those of *A. bernieri* (Randriamampianina et al., 2017) against the same sensitive bacteria.

There is no consensus on the standard scale for interpreting antimicrobial activity of natural products (Benko and Crovella, 2010). According to the scale of Dalmarco et al. (2010), used in this work, 2 bacteria were very sensitive, 3 moderately sensitive and 4 weakly sensitive. With standards used by other authors, plant extracts with MICs values higher than 500 $\mu\text{g/ml}$

(Aligianis et al., 2001) and even much higher than 1000 $\mu\text{g/ml}$ (Maregesi et al., 2008; Abubakar and Majinda, 2016; Touaibia and Chaouch, 2017) were classified as having strong antimicrobial activity. For comparison, LME and SME had excellent activities on all the bacteria tested.

Based on the results obtained with the strains tested, LME and SME have a relatively broad activity spectrum. The effect of SME on sensitive bacteria was bactericidal whereas SME exerted a bactericidal effect on some strains and bacteriostatic effect on others.

In conclusion, *Albizia masikororum* exerted antibacterial effect. LME and SME could constitute good alternatives to synthetic antibiotics, particularly against *Streptococcus*. Further studies should be undertaken to determine the number and the identities of active principles responsible of this property and to know whether or not they are new products.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

REFERENCES

- Abubakar MN, Majinda RRT (2016). Phytochemical constituents and antimicrobial activity of *Albizia Adianthifolia*. Journal of Pharmacognosy and Phytochemistry, 3(4):46-45.
- Agyare C, Koffuor GA, Mensah AY, Agyemang DO (2006). Antimicrobial and uterine smooth muscle activities of *Albizia ferruginea* extracts. Boletín Latinoamericano y del Caribe de Plantas Medicinales y Aromáticas, 5(2):31-35.
- Aligianis N, Kalpoutzakis E, Mitaku S, Chinou IJB (2001). Composition and antimicrobial activity of the essential oils of two *Origanum* species. Journal of Agricultural and Food Chemistry, 49(9):4168-4170.
- Ardanuy C, de la Campa AG, García E, Fenoll A, Calatayud L, Cercenado E, Pérez-Trallero E, Bouza E, Liñares J (2014). Spread of *Streptococcus pneumoniae* Serotype 8-ST63 Multidrug-Resistant Recombinant Clone, Spain. Emerging Infectious Diseases, 20(11):1848-1856.

- Benko IAM, Crovella S (2010). Ethnobotanical bioprospection of candidates for potential antimicrobial drugs from Brazilian plants. *Current Protein and Peptide Science*, 11(3):189-194.
- Bouharb H, El Badaoui K, Zair T, El amri J, Chakir S, Alaoui T (2014). Sélection de quelques plantes médicinales du Zerhoun (Maroc central) pour l'activité antibactérienne contre *Pseudomonas aeruginosa*. *Journal of Applied Biosciences*, pp. 6685-6693.
- Celikel N, Kavas G (2008). Antimicrobial properties of some essential oils against some pathogenic microorganisms. *Czech Journal of Food Sciences*, 26(3):174-181.
- Chamandi G, Olama Z, Holail H (2015). Antimicrobial effect of *Propolis* from different geographic origins in Lebanon. *International Journal of Current Microbiology and Applied Sciences*, 4(4):328-342.
- Dalmarco JB, Dalmarco EM, Koelzer J, Pizzolatti MG, Fröde TS (2010). Isolation and identification of bioactive compounds responsible for the anti-bacterial efficacy of *Lotus corniculatus* var. São Gabriel. *International Journal of Green Pharmacy*, 4:108-114.
- Djeussi DE, Noumedem JAK, Seukey J, Fankam AG, Voukeng IK, Tankeo SB, Nkuete AHL, Kuete V (2013). Antibacterial activities of selected edible plants extracts against multidrug-resistant Gram (-) bacteria. *BMC Complementary and Alternative Medicine*, 13:164.
- Kuete V, Fozing DC, Kapche WFGD, Mbaveng AT, Kuiate JR, Ngadjui BT, Abega ZBM (2009). Antimicrobial activity of the methanolic extract and compounds from *Morus mesozygia* stem bark. *Journal of Ethnopharmacology*, 124(3):551-555.
- Lu B, Fang Y, Fan Y, Chen X, Wang J, Zeng J, Zhang YLZ, Huang L, Li H, Li D, Zhu F, Cui Y, Wang D (2017). Macrolide-resistance and Molecular Characterization of *Streptococcus pyogenes* Isolates Circulating in China from 2009 to 2016. *Frontiers in Microbiology*, 8:1-10.
- Maregesi SM, Pieters L, Ngassapa OD, Apers S, Vingerhoets R, Cos P, Vanden BDA, Arnold JV (2008). Screening of some Tanzanian medicinal plants from Bunda district for antibacterial, antifungal and antiviral activities. *Journal of Ethnopharmacology*, 119:58-66.
- Marini-Bettolo GB, Nicoletti M, Patamia M, Galeffi C, Messana I (1981). Plant screening by chemical and chromatographic procedure under field conditions. *Journal of Chromatography A*, 213:113-127.
- McGaw LJ, Lall N, Hlokwé TM, Michel AL, Meyer JJ, Eloff JN (2008). Purified compounds and extracts from *Euclea* species with antimycobacterial activity against *Mycobacterium bovis* and fast-growing mycobacteria. *Biological and Pharmaceutical Bulletin*, 31(7):1429-1433.
- Nuermberger EL, Bishai WR (2004). Antibiotic resistance in *Streptococcus pneumoniae*: What does the future hold? *Clinical Infectious Diseases*, 38(Suppl 4): 363-371.
- Odeyemi O, Oluduro AO, David OM (2014). Evaluation of antimicrobial activities of *Albizia zygia* DC leaf extracts against some clinically important pathogens. *Journal of Natural Sciences Research*, 4(19):98-105.
- Ponce AG, Fritz R, del Valle C, Roura SI (2003). Antimicrobial activity of essential oils on the native microflora of organic Swiss chard. *Lebensmittel-Wissenschaft und -Technologie*, 36:679-684.
- Rakoto DAD, Rajemiarimoelisoa C, Randrianarivo R, Ramamonjison D, Raheriniaina C, Raharisoa N, Jeannoda V (2011). Antimicrobial Activity of Some Endemic Species of *Albizia* (FABACEAE) from Madagascar. *Asian Biotechnology and Development Review*, 13(3):53-60.
- Randriamampianina LJ, Razafintsalama VE, Rakoto DAD, Randrianarivo HR, Jeannoda VL (2017). Antimicrobial activity of seeds extracts from *Albizia bernieri* E. Fourn. (Fabaceae). *IOSR Journal of Pharmacy and Biological Sciences*, 12(3):72-79.
- Randrianarivo HR, Razafindrakoto AR, Ratsimanohatra HC, Randriamampianina LJ, Rajemiarimoelisoa CF, Ramamonjisoa L, Ramanitrahambola D, Rakoto DAD, Jeannoda VL (2014). Toxic effects of seed methanolic extracts of Endemic *Albizia* species (Fabaceae) from Madagascar on animals. *Journal of Life Sciences*, 8(8): 676-689.
- Roca M, Akova, Baquero F, Carlet J, Cavaleri M, Coenen S, Cohen J, Findlay D, Gyssens I, Heur OE, Kahlmeter G, Kruse H, Laxminarayan R, Liébana E, López-Cerero L, MacGowan A, Martins M, Rodríguez-Baño J, Rolain JM, Segovia C, Sigauque B, Taconelli E, Wellington E, Vila J (2015). The global threat of antimicrobial resistance: science for intervention. *New Microbes and New Infections*, 6:22-29.
- Salehi B, Ayatollahi SA, Segura-Carretero A, Kobarfard F, Contreras MDM, Faizi M, Sharifi-Rad M, Tabatabai SA, Sharifi-Rad J (2017). Bioactive chemical compounds in *Eremurus persicus* (Joub. & Spach) Boiss. essential oil and their health implications. *Cellular and Molecular Biology*, 63(9):1-7.
- Sarkiyayi S, Karago J, Hassan R (2011). Studies on anti-typhoid properties of aqueous methanol leaves extract of *Albizia ferruginea* (Musase). *International Journal of Current Microbiology and Applied Sciences*, 1(1): 24-30.
- Sekizuka T, Nai E, Yoshida T, Endo S, Hamajima E, Akiyama S, Ikuta Y, Obana N, Kawaguchi T, Hayashi K, Noda M, Sumita T, Kokaji M, Katori T, Hashino M, Oba K, Kuroda M (2017). Streptococcal toxic shock syndrome caused by the dissemination of an invasive emm3/ST15 strain of *Streptococcus pyogenes*. *BMC Infectious Diseases*, 17(774):1-4.
- Sharifi-Rad J (2016). Herbal antibiotics: Moving back into the mainstream as an alternative for "Superbugs". *Cellular and Molecular Biology*, 62(9) : 1-2.
- Sharifi-Rad J, Ayatollahi SA, Varoni EM, Salehi B, Kobarfard F, Sharifi-Rad M, Iriti M, Sharifi-Rad M (2017a). Chemical composition and functional properties of essential oils from *Nepeta schiraziana* Boiss. *Farmacia*, 65(5): 802-812.
- Sharifi-Rad J, Salehi B, Schnitzler P, Ayatollahi SA, Kobarfard F, Fathi M, Eisazadeh M, Sharifi-Rad M (2017b). Susceptibility of herpes simplex virus type 1 to monoterpenes thymol, carvacrol, p-cymene and essential oils of *Sinapis arvensis* L., *Lallemantia royleana* Benth. and *Pulicaria vulgaris* Gaertn. *Cell Molecule Biology*, 63(8) :42-47.
- Sharifi-Rad J, Salehi B, Varoni EM, Sharopov F, Yousaf Z, Ayatollahi SA, Kobarfard F, Sharifi-Rad M, Afdjei MH, Sharifi-Rad M, Iriti M (2017c). Plants of the *Melaleuca* genus as antimicrobial agents: From farm to pharmacy. *Phytotherapy Research*, 10:1475-1494.
- Sharifi-Rad J, Salehi B, Stojanović-Radić ZZ, Fokou PVT, Sharifi-Rad M, Mahady GB, Masjedi MR, Lawal TO, Ayatollahi SA, Masjedi J, Sharifi-Rad R, Setzer WN, Sharifi-Rad M, Kobarfard F, Rahman A, Choudhary MI, Ata A, Iriti M (2017d). Medicinal plants used in the treatment of tuberculosis - Ethnobotanical and ethnopharmacological approaches. *Biotechnology Advances*, S0734-9750(17)30077-0.
- Sharifi-Rad M, Varoni EM, Salehi B, Sharifi-Rad J, Matthews KR, Ayatollahi S, Kobarfard F, Ibrahim SA, Mnayer D, Zakaria ZA, Sharifi-Rad M, Yousaf Z, Iriti M, Basile A, Rigano D (2017e). Plants of the genus *Zingiber* as a source of bioactive phytochemicals: From tradition to pharmacy. *Molecules*, 22(12):2145.
- Touaibia M, Chaouch FZ (2017). Propriétés antioxydantes et antimicrobiennes des extraits de *Myrtus nivellei* Batt et *Trab. obtenus in situ et in vitro*. *Phytothérapie*, 15(1):16-22.
- Zhao C, Li Z, Zhang F, Zhang X, Ji P, Zeng J, Hu B, Hu Z, Liao K, Sun H, Zhang R, Cao B, Zhuo C, Jia W, Mei Y, Chu Y, Xu X, Yang Q, Jin Y, Fu Q, Xu X, Li H, Wang L, Ni Y, Liang H, Wang H (2017). Serotype distribution and antibiotic resistance of *Streptococcus pneumoniae* isolates from 17 Chinese cities from 2011 to 2016. *BMC Infectious Diseases*, 17(804): 1-12.

Full Length Research Paper

In-vitro antibiotic susceptibility profile of *Salmonella enterica* Serovar Typhi isolated from fecal specimens of humans in Umuahia metropolis, Abia State, Nigeria

Emmanuel O. Ekundayo^{1, 2*} and Emmanuel Enya²

¹Centre for Molecular Biosciences and Biotechnology, Michael Okpara University of Agriculture Umudike, Abia State, Nigeria.

²Department of Microbiology, College of Natural Sciences, Michael Okpara University of Agriculture Umudike, Abia State, Nigeria.

Received 24 March, 2018; Accepted 9 May, 2018

Typhoid is routinely diagnosed in Nigeria on clinical grounds or based on Widal serological test result. This approach does not provide information on the antibiotic susceptibility of the bacterium; *Salmonella enterica* Serovar Typhi. This study was done to assess the antimicrobial susceptibility profile of *Salmonella* isolates in Umuahia. In this study, seventy-two (72) *Salmonella* isolates obtained from 135 fecal specimens were tested for their antibiotic susceptibility profile against 13 antimicrobial agents using the disk diffusion method according to the protocol of the Clinical and Laboratory Standards Institute (CLSI). Sixty-two (86.1%) of the isolates were resistant to Amoxicillin, fifty-two (72.2%) to Trimethoprim-sulfamethoxazole (Co-trimoxazole), forty-one (56.9%) to Chloramphenicol and fifty-two (72.2%) to Augmentin. Fifty-nine (81.9%) of the isolates were resistant to Tetracycline, 11(15.2%) were of intermediate susceptibility and only two (2.7%) were susceptible to the antibiotic. Thirty-five (48.6%) of the isolates were sensitive to Ciprofloxacin, forty-seven (65.2%) to ofloxacin and forty-three (59.7%) to gentamicin. the resistance profile of the isolates to cephalosporin antibiotics was as follows: cefuroxime (70.8%), ceftriaxone (68.0%) and ceftazidime (65.2%). Some of the isolates exhibited resistance to multiple antibiotics ranging from three or more of the antibacterial agents tested. The results obtained suggest that high proportion of *S. Typhi* strains circulating in the study area are resistant to multiple antibiotics and empirical treatment of typhoid fever without antibiotic susceptibility testing is not advisable in this setting.

Key words: *Salmonella typhi*, antimicrobial resistance, typhoid fever, antibiotic susceptibility profile, Umuahia

INTRODUCTION

Typhoid fever is a life-threatening systemic febrile illness caused by the bacterium *Salmonella enterica* Serovar

Typhi (*S. Typhi*). Typhoid fever is a global public health problem with an estimated 21.7 million illnesses and

*Corresponding author. E-mail: ekundayo.emmanuel@mouau.edu.ng.

217,000 deaths worldwide in 2000 (Crump and Mintz, 2004). Antibiotic therapy has been the mainstay of management of typhoid fever but strains resistant to commonly used antibiotics have emerged and are spreading around the world (Zaki and Karande, 2011). Nigeria falls within the region of estimated high incidence of typhoid fever in Sub-Saharan Africa (Von Kalckreuth et al., 2016). The co-endemicity of typhoid fever along with other non-bacterial febrile infections like malaria and viral diseases makes direct clinical diagnosis difficult (Enabulele and Awunor, 2016).

For confirmation of diagnosis of typhoid fever and for effective antibiotic therapy, there is a need to isolate *S. Typhi* and carry out antibiotic susceptibility testing. Isolation of *S. Typhi* from culture of bone marrow aspirate is regarded as the gold standard for confirmation of typhoid fever but blood culture is preferred because obtaining blood specimen is easier and less invasive (Bhutta, 2006). Blood culture is slightly less sensitive than bone marrow aspirate culture (Tanyigna et al., 2001). Stool culture can be done as an alternative to blood culture. However, culture for isolation of *S. Typhi* is rarely done in low income countries due to high cost, lack of equipment and technical expertise. As a result, data on the prevalence and pattern of resistance to antimicrobials among strains of *S. Typhi* are limited in low income countries (Von Kalckreuth et al., 2016). Available data indicate that strains of *S. Typhi* resistant to multiple antibiotics have been isolated in some parts of Nigeria (Akinyemi et al., 2014).

In Nigeria, typhoid fever is typically diagnosed on clinical grounds or based on the results of Widal serological test and antibiotic treatment is done empirically with no certainty about the sensitivity of the infecting pathogen. Widal test has the problem of low sensitivity and specificity with positive predictive values (PPV) around 17-20% (Tanyigna et al., 2001; Enabulele and Awunor, 2016). In view of the low PPV of Widal test and the uncertainty associated with the antibiotic susceptibility profile of *S. Typhi* strains, many patients are likely to receive inappropriate antibiotic therapy in the absence of culture and antibiotic susceptibility testing.

Increasing rates of reduced susceptibility to ciprofloxacin commonly used in cases of suspected resistance to first line antimicrobials have been reported (Harrois et al., 2014; Lunguya et al., 2013; Kariuki et al., 2010, Akinyemi et al., 2007). Antibiotic susceptibility surveillance systems can provide insights into the areas where resistance is most prevalent and where the prevalence of resistance is increasing the fastest. Determination of antimicrobial susceptibility patterns not only helps shape successful treatment plans for individual patients but also assists with the development of public health policy for control of antimicrobial resistance. The aim of this study was to determine the antibiotic susceptibility profile of strains of *Salmonella* isolated from fecal specimens of patients in Umuahia.

MATERIALS AND METHODS

Specimen collection and inoculation

Fecal specimens were collected from patients attending four different clinics in Umuahia metropolis, the Capital of Abia State. Each of the patients that submitted specimens gave an informed consent. Only one fecal specimen was collected per participant. The management of each of the clinics gave approval for the collection of specimens. The specimens were inoculated within 24 h of collection into Selenite Faeces broth (Titan Biotech Ltd, India) for enrichment and incubated at 37°C for 18 - 24 h and subsequently plated on Salmonella-Shigella agar (Titan Biotech Ltd, India) for primary isolation. The culture plates were incubated aerobically at 37°C for 24 h and observed for growth through the formation of colonies. Colonies growing on the plates were purified by streak plate method on nutrient agar and subsequently maintained on nutrient agar slants.

Biochemical characterization of isolates

Standard microbiological techniques were performed for biochemical characterization of the isolates. Biochemical tests carried out according to standard procedures as described in Cheesbrough (2006), included Indole, Citrate, Urease, Methyl Red, Voges Proskauer and Triple Sugar Iron tests.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed using the Kirby-Bauer disk agar diffusion method according to the Clinical and Laboratory Standards Institute (CLSI) protocol (CLSI, 2015) on Mueller Hinton agar (Hardy Diagnostics, USA). Discrete colonies from a 24 h nutrient agar plates were suspended in sterile normal saline in a test tube to achieve a bacterial suspension equivalent to 0.5 McFarland Turbidity Standard. A cotton swab was dipped into the bacterial suspension and used to inoculate the entire surface of a Mueller Hinton agar plate, rotating the plate to ensure confluent growth of the bacterium. The antimicrobial susceptibility disks were placed on the surface of the inoculated plate with flame sterilized forceps. The plates were incubated in an inverted position for 16–18 h at 35-37°C. The diameter of the zone of inhibition produced by the antibiotic disks were measured to the nearest millimeter (mm) using a transparent ruler.

Data and statistical analysis

The criteria for categorizing the diameter of zones of inhibition into sensitive (S) or intermediate (I) or resistant (R) were based on the interpretive charts of the Clinical and Laboratory Standards Institute (CLSI, 2015). Proportions of males and females and resistant isolates were compared using the Pearson's approximation to the Chi-squared test. The significance level was tested at $\alpha = 0.05$.

RESULTS

Fecal specimens were collected from a total of 135 study participants, made of 59 (43.7%) males and 76 (56.3%) females. The demographic characteristics of the study participants and the number of *Salmonella* isolates recovered according to gender and age groups are shown in Table 1. Difference between the number of

Table 1. Demographic characteristics of study participants and isolation of *Salmonella* from fecal specimens.

Characteristics	No. (%) of fecal specimens	No. yielding <i>Salmonella</i> isolates	Isolation rate (%)
Sex			
Male	59 (43.70)	30	22.20
Female	76 (56.30)	42	31.10
Total	135 (100)	72	53.30
Age groups (years)			
10-20	14 (10.40)	9	6.67
21-30	63 (46.70)	39	28.9
31-40	30 (22.20)	12	8.99
41-50	15 (11.10)	9	6.67
51-60	11 (8.00)	3	2.22
>61	2 (1.50)	0	0.00

Table 2. Antimicrobial agents tested and criteria for assessment of susceptibility of *Salmonella* isolates.

Antimicrobial agents (code) ^a	Potency (µg)	Inhibition zone (mm) cut-off values*		
		(S)	(I)	(R)
Gentamicin (GEN)	10	≥15	13-14	≤12
Chloramphenicol (CHL)	30	≥18	13-17	≤11
Ofloxacin (OFL)	30	≥16	13-15	≤12
Augmentin (AUG)	30	≥18	14-17	≤13
Ciprofloxacin (CPR)	5	≥21	16-20	≤15
Amoxicillin (AMX)	25	≥18	14-15	≤16
Cefuroxime (CXM)	30	≥18	15-17	≤14
Cotrimoxazole (COT)	25	≥16	11-15	≤10
Ceftazidime (CAZ)	30	≥21	18-20	≤17
Nitrofurantoin (NIT)	100	≥17	15-16	≤14
Ceftriaxone (CRO)	30	>23	20-22	<19
Tetracycline (TET)	30	≥15	12-14	≤11
Streptomycin (STR)	25	≥15	12-14	≤11

*Interpretative break points according to Clinical Laboratory Standards Institute, CLSI (2015). a, Abtek™ Biologicals, UK.

males and females that submitted fecal specimens was not significantly different ($p>0.05$). The difference between the number of *Salmonella* isolates recovered from males and females was however, significantly different ($p<0.01$). *Salmonella* was isolated from 53.3% of the total number of participants who submitted fecal specimens.

The antimicrobial agents tested and the interpretative criteria for assessment of the susceptibility of *Salmonella* based on the diameter of zones of inhibition of each antimicrobial agent are presented in Table 2. The antibiotic susceptibility profile showing the proportions of Sensitive, Intermediate and Resistant categories is presented in Figure 1. The isolates exhibited high resistance level to amoxicillin, augmentin, trimethoprim sulfamethoxazole (co-trimoxazole) tetracycline, chloramphenicol and the second and third generations

cephalosporins. Sensitivity of the isolates to ofloxacin and gentamicin was moderate. Greater than 30% of the isolates have reduced sensitivity (intermediate category) to Chloramphenicol and Ciprofloxacin.

The isolates were assessed for resistance to multiple antimicrobial agents, from combination of three agents to seven. The results are shown in Table 3. Six patterns of resistance to combination of three antimicrobial agents were identified. About 52% of the isolates were resistant to combinations of three different types of antimicrobial agents and about 35% were resistant to combinations of four antimicrobial agents. The commonest multidrug resistant (MDR) phenotypes are amoxicillin, chloramphenicol and tetracycline (14 isolates), followed by amoxicillin, chloramphenicol, tetracycline and cotrimoxazole (12 isolates). The classical *Salmonella* MDR phenotype defined by resistance to amoxicillin,

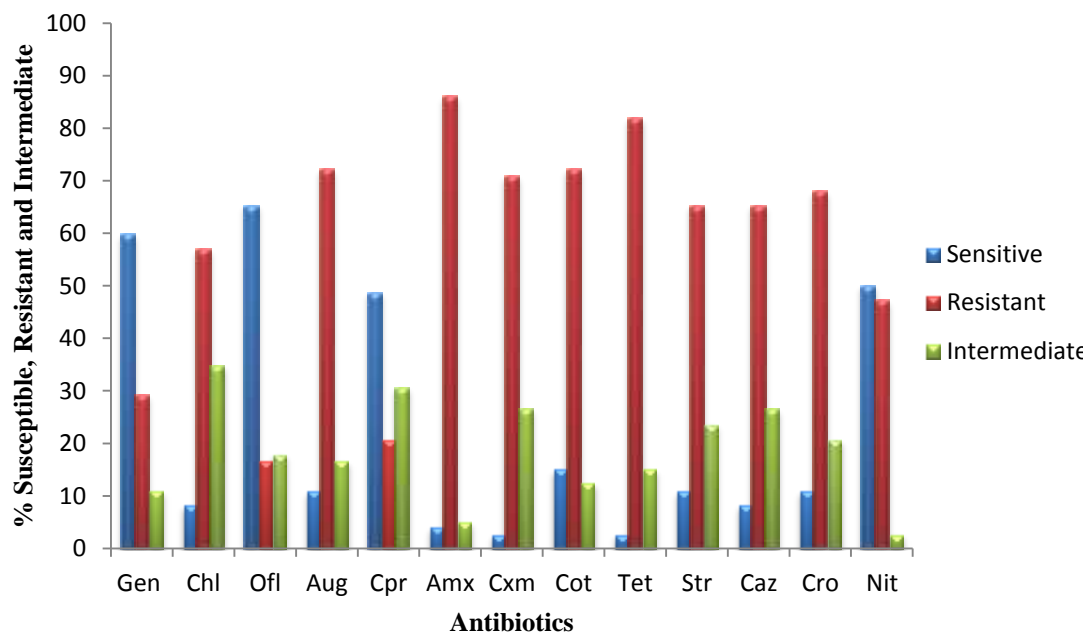


Figure 1. Antimicrobial susceptibility profile of salmonella isolates in Umuahia. Gentamicin (Gen), chloramphenicol (Chl), ofloxacin (Ofi), augmentin (Aug), ciprofloxacin (Cpr), amoxicillin (Amx), cefuroxime (Cxm), ceftriaxone (Ctx), tetracycline (Tet), streptomycin (Str), ceftazidime (Caz), cotrimoxazole (Crx), nitrofurantoin (Nit).

Table 3. Multiple antibiotic resistance profile of the isolates.

Antibiotics	No. of antibiotics	No. of antibiotic resistant isolates	Overall (%)
AXM, CHL, COT	3	9	
AMX, CHL, TET	3	14	
AMX, TET, GEN	3	1	38 (52.8)
AMX, TET, CPR	3	3	
AMX, CHL, CPR	3	2	
AMX, AUG, COT	3	9	
AMX, CHL, COT, TET	4	12	
AMX, AUG, GEN, CAZ	4	3	
AMX, AUG, OFL, COT	4	1	23 (31.9)
AMX, CHL, COT, CPR	4	4	
AMX, AUG, GEN, CAZ	4	2	
AMX, AUG, OFL, COT	4	1	
AMX, AUG, GEN, OFL, CHL	5	1	
AMX, TET, CRO, OFL, CHL	5	2	
AMX, CPR, CRO, OFL, NIT	5	1	4 (6.15)
AMX, CPR, COT, TET, NIT, GEN	6	1	
AMX, COT, CAZ, CRO, AUG, CPR	6	5	8 (12.30)
AMX, AUG, CRO, OFL, NIT, GEN	6	2	
AMX, AUG, CRO, CPR, NIT, STR, CHL	7	1	1 (1.53)
Number of MDR Patterns	18	72	90.3%

Gentamicin (GEN), chloramphenicol (CHL), ofloxacin (OFL), augmentin (AUG), ciprofloxacin (CPR), amoxicillin (AMX), cefuroxime (CXM), ceftriaxone (CRO), tetracycline (TET), streptomycin (STR), ceftazidime (CAZ), cotrimoxazole (COT), nitrofurantoin (NIT).

chloramphenicol and co-trimoxazole was observed in nine isolates. The number of isolates resistant to amoxicillin, chloramphenicol and ciprofloxacin was relatively small (only two isolates).

DISCUSSION

From the 135 fecal specimens cultured, 72 yielded *Salmonella* organisms representing an isolation rate of 53.3%. This is similar to that of Duthie and French (1990) who reported an isolation rate of 59%. The sensitivity of stool culture is believed to be lower than that of blood culture which is recommended as the gold standard for confirmation of diagnosis of typhoid (Tanyigna et al., 2001; Ameya et al., 2017). However, blood culture is more difficult and requires more expensive laboratory infrastructure and greater technical expertise (Von Kalkreuth et al., 2016). Although, stool culture may not give accurate incidence rate, the results of this study suggest that stool culture can yield adequate number of isolates for monitoring antimicrobial susceptibility profile of *Salmonella* from humans. In this study, we found a significant difference between number of isolates from male and female study participants. This is consistent with the findings of others (Ja'afar et al., 2013; Yaseen et al., 2017). The explanation for this is beyond the scope of the present work. It could be that the females are more susceptible to infection with *Salmonella* or more exposed to sources of infection. This requires further epidemiological study.

In this study, the antibiotic susceptibility profile of *Salmonella* isolates from fecal specimens of males and females indicate that strains of *Salmonella* resistant to first line drugs are common in the study area. However, we did not find strains that were completely resistant to all the antimicrobial agents. The isolates with multidrug resistant (MDR) phenotype were still highly susceptible to ciprofloxacin although considerable number of strains has developed resistance to ciprofloxacin without the MDR phenotype. The prevalence of isolates with reduced susceptibility to ciprofloxacin is also high. Resistance to the fluoroquinolones typically evolves in a stepwise fashion (Rahman et al., 2014). This means that prolonged and continuous abuse of this antibiotic could lead to full resistance to the antibiotic. This will greatly limit the treatment options for the isolates that have developed resistance to the first line drugs. The susceptibility of the isolates to ofloxacin and gentamicin stood at 65.2 and 59.7%, respectively. These antibacterial agents are not commonly used for treatment of typhoid but in an environment where self-medication is rampant and abuse of antibiotics is common, the organisms might have acquired resistance to these agents under antibiotic pressure or through horizontal transfer of resistance genes.

The results of this study indicate that *Salmonella*

isolates from humans have developed resistance to common antibiotics such as amoxicillin, chloramphenicol, co-trimoxazole and this resistance has also spread to the fluoroquinolones. The decrease in the susceptibility of the *Salmonella* isolates to ofloxacin and ciprofloxacin has been reported in other parts of Nigeria (Akinyemi et al., 2014). Increased MDR has been reported among *Salmonella* isolates in many countries including Iran (Butaye et al., 2006) and Ethiopia (Zewdu and Cornelius, 2009). A previous study in Nigeria by Akinyemi et al. (2007) had reported a multi-drug resistance rate of 61.0%. The result of this study, however, revealed a comparatively high proportion of multidrug resistance with a prevalence rate of up to 90.3%.

The prevalence of multi-drug resistance reported in this study reveals a limitation of an effective treatment of human *Salmonella* infection with commonly used antibiotics. The antimicrobial susceptibility profile of *Salmonella* isolates from humans reported in this study suggests that effective treatment of typhoid fever cannot likely be achieved without antibiotic susceptibility testing of individual isolates. Therefore, diagnosis of typhoid fever by non-cultural methods and empirical antibiotic therapy without information on the antibiotic susceptibility of the causative agents are no longer appropriate. In addition, this study indicates a need for continuous monitoring of antibiotic susceptibility pattern of *Salmonella* isolates in Umuahia and the neighborhood.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

REFERENCES

- Akinyemi KO, Bamiro BS, Coker AO (2007). Salmonellosis in Lagos, Nigeria: Incidence of *Plasmodium falciparum*-associated co-infection, patterns of antimicrobial resistance, and emergence of reduced susceptibility to fluoroquinolones. *Journal of Health, Population and Nutrition*, 25(3):351-358.
- Akinyemi KO, Iwalokun BA, Agboyinu JA, Ogunyemi O, Fasure AK (2014). Emergence of Third Generation Cephalosporin Resistance and Typing by Randomly Amplified Polymorphic DNA (RAPD) among Clinical *Salmonella* Isolates from Lagos, Nigeria. *British Microbiology Research Journal*, 4(6): 668-677.
- Ameya G, Atalel E, Kebede B, Yohanne B (2017). Comparative Study of Widal test against stool culture for typhoid fever suspected cases in Southern Ethiopia. *Pathology and Laboratory Medicine International*, 9:1-7.
- Bhutta ZA (2006). Current concepts in the diagnosis and treatment of typhoid fever. *British Medical Journal*, 333:78-82.
- Butaye P, Michael GB, Schwarz S, Barrett TJ, Brisabois A, White DG (2006). The clonal spread of multidrug-resistant non-typhi *Salmonella* serotypes. *Microbes and Infection*, 8:1891-1897.
- Cheesbrough M (2006). *District Laboratory Practice in Tropical Countries (Part 2)*. Cambridge University Press Edinburgh, UK. pp. 62-70.
- CLSI (2015). Performance standards for Antimicrobial Susceptibility Testing Twenty Fifth Informational Supplement. CLSI document M100-S25 Wayne, PA: Clinical and Laboratory Standards Institute.
- Crump JA, Mintz ED (2004). The global burden of typhoid fever. *Bulletin of the World Health Organization*, 82(5):346-353.

- Duthie R, French GL (1990). Comparison of methods for the diagnosis of typhoid fever. *Journal of Clinical Pathology*, 43:863-865.
- Enabulele O, Awunor SN (2016). Typhoid fever in a Tertiary Hospital in Nigeria: Another look at the widal agglutination test as a preferred option for diagnosis. *Nigerian Medical Journal*, 57(3):145-149
- Harrois D, Breurec S, Seck A, Delaune A, LeHello S, Pardosde la Gándara M (2014). Prevalence and characterization of extended-spectrum beta-lactamase producing clinical *Salmonella enterica* isolates in Dakar, Senegal, from 1999 to 2009. *Clinical Microbiology and Infection*, 20:109-116.
- Ja'afar NJ, Goay YX, Zaidi NFM, Low HC, Husain HM, Hamzah WM, Bhore SJ, Balaram P, Ismail A, Phua KK (2013). Epidemiological analysis of typhoid fever in Kelantan from a retrieved registry. *Malaysian Journal of Microbiology*, 9(2):147-151.
- Kariuki S, Revathi G, Kiiru J (2010). Typhoid in Kenya is associated with a dominant multidrug-resistant *Salmonella enterica* serovar Typhi haplotype that is also widespread in Southeast Asia. *Journal of Clinical Microbiology*, 48:2171-76.
- Lunguya O, Lejon V, Phoba MF, Bertrand S, Vanhoof R, Glupczynski Y, Verhaegen J, Muyembe-Tamfum JJ, Jacobs J (2013). Antimicrobial resistance in invasive non-typhoid *Salmonella* from the Democratic Republic of the Congo: emergence of decreased fluoroquinolone susceptibility and extended-spectrum beta lactamases. *PLoS Neglected Tropical Diseases*, 7(3):e2103.
- Rahman BA, Wasfy MO, Maksoud MA, Hanna N, Dueger E, House B (2014). Multi-drug resistance and reduced susceptibility to ciprofloxacin among *Salmonella enterica* serovar Typhi isolates from the Middle East and Central Asia. *New Microbe and New Infections*, 2:88-92.
- Tanyigna KB, Bello CS, Okeke N, Onwukeme KE (2001). Comparison of blood, bone marrow aspirate, stool and urine cultures in the diagnosis of enteric fever. *Nigerian Journal of Medicine*, 10:21-24.
- Von Kalckreuth V, Konings F, Aaby P, Adu-Sarkodie Y, Ali M, Aseffa A et al. (2016). The typhoid fever surveillance in Africa Programme (TSAP): Clinical, diagnostics, and epidemiological methodologies. *Clinical Infectious Diseases*, 62(1): S9-S16.
- Yaseen KM, Abdul J, Afshan S, Kifayat U, Ur RH, Osman A, Nasir H, Adeel A (2017). Endemic study of typhoid fever between male and female population of Ali Pur, Islamabad. *BioMedicine and Surgery*, 1(3):105-108.
- Zaki SA, Karande S (2011). Multidrug resistant typhoid fever. A review. *The Journal of Infection in Developing Countries*, 5:324-337.
- Zewdu E, Cornelius P (2009). Antimicrobial resistance of *Salmonella* serotypes isolated from food items and personnel in Addis Ababa, Ethiopia. *Tropical Animal Health and Production*, 41:241-249.

OPEN ACCESS



African Journal of
Microbiology Research

21 May 2018
ISSN 1996-0808
DOI: 10.5897/AJMR
www.academicjournals.org

 **ACADEMIC
JOURNALS**
expand your knowledge

ABOUT AJMR

The African Journal of Microbiology Research (AJMR) is published weekly (one volume per year) by Academic Journals.

The African Journal of Microbiology Research (AJMR) provides rapid publication (weekly) of articles in all areas of Microbiology such as: Environmental Microbiology, Clinical Microbiology, Immunology, Virology, Bacteriology, Phycology, Mycology and Parasitology, Protozoology, Microbial Ecology, Probiotics and Prebiotics, Molecular Microbiology, Biotechnology, Food Microbiology, Industrial Microbiology, Cell Physiology, Environmental Biotechnology, Genetics, Enzymology, Molecular and Cellular Biology, Plant Pathology, Entomology, Biomedical Sciences, Botany and Plant Sciences, Soil and Environmental Sciences, Zoology, Endocrinology, Toxicology. The Journal welcomes the submission of manuscripts that meet the general criteria of significance and scientific excellence. Papers will be published shortly after acceptance. All articles are peer-reviewed.

Contact Us

Editorial Office: ajmr@academicjournals.org

Help Desk: helpdesk@academicjournals.org

Website: <http://www.academicjournals.org/journal/AJMR>

Submit manuscript online <http://ms.academicjournals.me/>

Editors

Prof. Adriano Gomes da Cruz
*University of Campinas (UNICAMP),
Brazil.*

Prof. Ashok Kumar
*School of Biotechnology
Banaras Hindu University Uttar Pradesh,
India.*

Dr. Mohd Fuat Abd Razak
*Infectious Disease Research Centre,
Institute for Medical Research, Jalan
Pahang, Malaysia.*

Dr. Adibe Maxwell Ogochukwu
*Department of Clinical Pharmacy and
Pharmacy Management,
University of Nigeria
Nsukka, Nigeria.*

Dr. Mehdi Azami
*Parasitology & Mycology Department
Baghaeei Lab.
Isfahan, Iran.*

Dr. Franco Mutinelli
*Istituto Zooprofilattico Sperimentale delle
Venezie Italy.*

Prof. Ebiamadon Andi Brisibe
*University of Calabar,
Calabar,
Nigeria.*

Prof. Nazime Mercan Dogan
*Department of Biology
Faculty of Science and Arts
University Denizli Turkey.*

Prof. Long-Liu Lin
*Department of Applied Chemistry
National Chiayi University
Chiayi County Taiwan.*

Prof. Natasha Potgieter
*University of Venda
South Africa.*

Dr. Tamer Edirne
*Department of Family Medicine
University of Pamukkale
Turkey.*

Dr Kwabena Ofori-Kwakye
*Department of Pharmaceutics
Kwame Nkrumah University of Science &
Technology
Kumasi, Ghana.*

Dr. Tülin Askun
*Department of Biology
Faculty of Sciences & Arts
Balikesir University Turkey.*

Dr. Mahmoud A. M. Mohammed
*Department of Food Hygiene and Control
Faculty of Veterinary Medicine
Mansoura University Egypt.*

Editors

Dr. James Stefan Rokem
Department of Microbiology & Molecular Genetics
Institute of Medical Research Israel – Canada
The Hebrew University – Hadassah Medical School Jerusalem, Israel.

Dr. Afework Kassu
University of Gondar
Ethiopia.

Dr. Wael Elnaggar
Faculty of Pharmacy
Northern Border University
Rafha Saudi Arabia.

Dr. Maulin Shah
Industrial Waste Water Research Laboratory
Division of Applied & Environmental Microbiology, Enviro Technology Limited
Gujarat, India.

Dr. Ahmed Mohammed
Pathological Analysis Department
Thi-Qar University College of Science
Iraq.

Prof. Naziha Hassanein
Department of Microbiology
Faculty of Science
Ain Shams University
Egypt.

Dr. Shikha Thakur
Department of Microbiology
Sai Institute of Paramedical and Allied Sciences India.

Dr. Samuel K Ameyaw
Civista Medical Center
USA.

Dr. Anubrata Ghosal
Department of Biology
MIT - Massachusetts Institute of Technology
USA.

Dr. Bellamkonda Ramesh
Department of Food Technology
Vikrama Simhapuri University
India.

Dr. Sabiha Yusuf Essack
Department of Pharmaceutical Sciences
University of KwaZulu-Natal
South Africa.

Dr. Navneet Rai
Genome Center
University of California Davis USA.

Dr. Iheanyi Omezuruike Okonko
Department of Virology
Faculty of Basic Medical Sciences
University of Ibadan
Ibadan, Nigeria.

Dr. Mike Agenbag
Municipal Health Services,
Joe Gqabi,
South Africa.

Dr. Abdel-Hady El-Gilany
Department of Public Health & Community Medicine, Faculty of Medicine
Mansoura University
Egypt.

African Journal of Microbiology Research

Table of Content: Volume 12 Number 19 21 May, 2018

ARTICLES

- Molecular characterization of *Listeria monocytogenes* isolated from a ready-to-eat fermented milk and cereal product, *Fura-de-Nunu*** **448**
EZEONU Ifeoma M., ONONUGBO Chukwuebuka M. and
IKE Anthony C.
- Emphasis on functional properties of cocoa-specific acidifying lactic acid bacteria for cocoa beans fermentation improvement** **456**
Evelyne Cho Adiko, Ginette Gladys Doué, Lessoy Thierry Zoué and
Sébastien Lamine Niamké

Full Length Research Paper

Molecular characterization of *Listeria monocytogenes* isolated from a ready-to-eat fermented milk and cereal product, *Fura-de-Nunu*

EZEONU Ifeoma M.*, ONONUGBO Chukwuebuka M. and IKE Anthony C.

Department of Microbiology, University of Nigeria, Nsukka, Enugu State, Nigeria.

Received 3 March, 2018; Accepted 18 May, 2018

This study was conducted to determine the occurrence of *Listeria (L.) monocytogenes* in *Fura-de-Nunu*, a ready-to-eat (RTE) fermented milk (*Nunu*) and cereal (*Fura*) blend, the serogroups as well as the virulence of the isolates. A total of 75 *Fura* and 75 *Nunu* samples were examined. *Listeria* species were isolated on PALCAM medium and *Listeria* chromogenic agar, and identified phenotypically according to International Standardization Organization (ISO) procedures. Identification of *L. monocytogenes*, serogrouping and detection of virulence genes were carried out by polymerase chain reaction (PCR). *Listeria* spp. were recovered from 23 (30.67%) and 41 (54.67%) samples of *Fura* and *Nunu*, respectively. The bioloads of *Listeria* ranged from 10^3 to 10^5 CFU/ml. Six presumptive species of *Listeria* were identified from the samples, with *L. monocytogenes* accounting for 21.00 and 20.64% of isolates from *Fura* and *Nunu*, respectively. Out of the three major serogroups (1/2a, 1/2b and 4b) associated with human disease, only 1/2a and 4b were identified among the isolates. Some of the isolates tested positive for the presence of virulence genes, *hlyA* and *iap*. Results from this study show that *Fura-de-Nunu*, may represent a risk for transmission of listeriosis to consumers.

Key words: *Listeria monocytogenes*, *Fura-de-Nunu*, fermented milk, ready-to-eat, Listeriosis.

INTRODUCTION

The genus *Listeria* currently comprises seventeen species of Gram-positive, non-spore forming, and catalase-positive bacteria: *Listeria (L.) aquatica*, *L. booriae*, *L. cornellensis*, *L. fleischmannii*, *L. floridensis*, *L. grandensis*, *L. grayi*, *L. innocua*, *L. ivanovii*, *L. marthii*, *L. monocytogenes*, *L. newyorkensis*, *L. riparia*, *L. rocourtiae*, *L. seeligeri*, *L. weihenstephanensis*, and *L. welshimeri* (Weller et al., 2015). The haemolytic species

(*L. monocytogenes*, *L. ivanovii* and *L. seeligeri*) are associated with human disease, although *L. ivanovii* and *L. seeligeri* are more commonly involved in diseases of animals and are rare causes of human disease (Guillet et al., 2010; Jamali et al., 2013; Abdellrazeq et al., 2014).

L. monocytogenes has emerged as a very important food-borne pathogen, causing the human disease, listeriosis (Marnissi et al., 2013; Mansouri-Najand et al.,

*Corresponding author. E-mail: ifeoma.ezeonu@unn.edu.ng. Tel: +234 803 795 4649.

2015). It has been detected in various foods, including raw milk and other dairy products, such as cheese and ice cream (Jamali et al., 2013). Infectious doses are reported to range from 10^1 to 10^5 CFU/g of food (Maijala et al., 2001; Ooi and Lorber, 2005; Yang et al., 2007; Marnissi et al., 2013; Jamali et al., 2013; Mansouri-Najand et al., 2015; Oyinloye, 2016). However, according to the European food safety regulations, generally, food samples containing more than 100 CFU/g of *L. monocytogenes* are considered unsatisfactory. In foods intended for infants or foods where the shelf life is determined without consideration of the organism, there should be complete absence of *L. monocytogenes* /25 g of the food before the food leaves the immediate control of the producer (Food Standards Agency, 2006; Public Health England, 2014). Listeriosis is a disease of serious public health concern because of the high mortality rate associated with the disease; especially in pregnant women and infants, the elderly and immunocompromised individuals, who are at the greatest risk for the disease (Gillespie et al., 2010; Barton et al., 2011; Abdellrazeq et al., 2014; Oyinloye, 2016). A survey in 2010, by the Europe PMC Funders Group, estimated that listeriosis resulted in 23,150 illnesses, 5,463 deaths and 172, 823 disability adjusted life years (DALYs) globally. The proportion of perinatal cases was 20.7%. This led to a call by the World Health Organization (WHO) for more studies, particularly in developing countries (de Noordhout et al., 2014).

The presence of *L. monocytogenes* in ready-to-eat (RTE) foods and the low infectious dose of the organism are a cause for concern as such foods could constitute health hazards to consumers. *L. monocytogenes* has been reported from humans, environment and various foods in Nigeria, although most of these reports have come from studies based on phenotypic and biochemical characterization of the organism (Nwaiwu, 2015). *Fura-de-Nunu*, a fermented milk-cereal mix, is a street-vended, RTE food, consumed in various parts of Nigeria, especially in Northern Nigeria. In Nigeria, about 90% of the dairy cattle belong to the Fulani agro-pastoralists and their women strictly control the processing and marketing of their milk products (Chukwuma, 2009; Okeke et al., 2014). Most of these women are illiterate and have no knowledge of critical control points of food production. *Fura* is a semi-solid dumpling millet-based meal. The millet is blended with spices and water, molded into dough balls and then cooked. To make the complete meal, *Fura-de-Nunu*, the cooked dough balls are broken up and mixed into the fermented milk (*Nunu*), to form porridge (Kordylasi, 1990; Jideani et al., 2001).

Fura-de-Nunu, like many other indigenous fermented foods, depends on spontaneous fermentation initiated by natural microorganisms on raw materials and equipment, the hands of producers and the local environment. In addition, some aspects of the production of this widely consumed product, such as use of leftover products as

starter for fresh fermentation of the *Nunu* and molding of the *Fura* by hand, all increase the chances of contamination of the product. This study therefore aimed to determine the prevalence of *L. monocytogenes* in RTE *Fura-de-Nunu* samples, the PCR serogroups as well as virulence genes of the isolates.

MATERIALS AND METHODS

Samples collection

A total of 150 samples, comprising 75 *Fura* and 75 *Nunu*, were purchased from vendors in different communities within Nsukka metropolis. *Nunu* samples were collected in a sterile large screw capped bottles while *Fura* balls were placed in sterile polyethylene bags and transported in ice-packed containers to the Microbiology Laboratory, University of Nigeria, Nsukka for analysis.

Isolation and enumeration of organisms

The procedure recommended by the International Organization for Standardization (ISO) 11290 (2004) was used as follows: A 25 g representative portion from each *Fura* sample, mashed into a semi-powdery paste or 25 mL representative portion of each *Nunu* sample was introduced aseptically into a sterile conical flask containing 225 mL of Half Fraser Broth to obtain a 1:10 sample dilution. The samples were homogenized for 1 min and incubated for 24 h at 37°C. After incubation, a 0.1 mL aliquot from each Half Fraser Broth culture was added to 9.9 mL of Fraser Broth and incubated for 24 to 48 h at 37°C. Following incubation, the culture was serially diluted ten-fold and 0.1 mL from 10^{-3} , 10^{-4} and 10^{-5} dilutions was spread on *Listeria* selective agar (PALCAM; Oxoid, UK). Samples were also inoculated onto plates of Brilliance *Listeria* chromogenic agar (Oxoid, UK), selective for *Listeria* spp. The plates were incubated for 24 to 48 h at 37°C and examined for grayish colonies, typical of *Listeria*. Total viable counts were taken from plates containing countable colonies.

Biochemical confirmation of the isolates

Colonies showing typical morphology of *Listeria* were streaked onto Tryptone Soya Agar supplemented with 0.6% yeast extract powder (TSYEA; Oxoid, UK) and incubated at 37°C for 24 h to further obtain pure cultures for identification and characterization (Alsheikh et al., 2013). The isolates were identified and characterized using the following tests: Gram staining, oxidase, catalase, motility test, sugar fermentation (Glucose, Rhamnose, Mannitol, methyl-D-mannose, xylose), and haemolysis test. Results from suspected *L. monocytogenes* isolates were compared to those of a reference *L. monocytogenes* strain ATCC13932

Haemolysis test

The isolates were inoculated onto 7% Sheep Blood Agar (SBA) by piercing into the medium and incubated at 37°C for 24 h. The haemolytic zones around the colonies were examined after incubation (Alsheikh et al., 2013; Momtaz and Yadollahi, 2013).

Christie, Atkins, Munch-Petersen (CAMP) test

This was performed according to the procedure described by Yadav et al. (2010). A β -haemolytic strain of *Staphylococcus aureus* was

grown overnight on 7% SBA at 37°C and a colony was streaked again on freshly prepared 7% SBA in a manner that the streak allows for streaking of *Listeria* colonies. *Listeria* isolates was streaked at 90° angle and 3 mm apart before incubating them at 37°C for 24 h. A positive reaction was indicated by an enhanced zone of haemolysis at the intersection of the test and indicator strain.

Determination of pH of samples

For the *Fura* samples, 10 g of each sample was homogenized with 20 mL of distilled water in a beaker, for 1 min and the pH of the homogenate determined using digital pH meter calibrated with standard buffer solutions. For the *Nunu* samples, the samples were mixed properly and 25 mL of each sample was poured into a beaker and the pH determined using a digital pH meter.

Determination of titratable acidity of the samples (using phenolphthalein indicator)

Ten millilitres (*Nunu*) or 10 g (*Fura*) of each sample was added to 50 mL of distilled water in a 250 mL beaker and homogenized properly. Then, 3 drops of phenolphthalein were added to the mixture. A 0.1 M solution of NaOH was slowly titrated into the mixture, while carefully swirling the beaker to ensure thorough mixing. The point of neutrality was reached when the indicator changed colour from colourless to pink, which must remain stable for at least 30 s. The amount of NaOH used on the burette was recorded and result expressed as percentage acid, using the following formula:

$$\text{Percent acid} = \frac{\text{Titre} \times \text{acid factor} \times 100}{10 \text{ mL of sample}}$$

Acid factor for Lactic acid = 0.0090

Determination of NaCl (salt content) with standard silver nitrate solution

To each sample neutralized as described above, about 1 mL of 5% aqueous potassium chromate solution was added and titrated with 0.1 N AgNO₃ solution to produce red-brown end point.

$$\text{NaCl \%} = \frac{\text{Titre value} \times \text{Normality of AgNO}_3 \times 58.4 \times 100}{\text{Weight of the sample} \times 1000}$$

Determination of moisture content

About 5 mL and 5 g of well mixed samples (*Nunu* and *Fura*) were weighed in a well dried oven sterilized glass tray. The tray was placed in a hot air oven maintained at 105 ± 2°C and dried for at least 2 h. It was then cooled in a dessicator and weighed. The process of heating, cooling and weighing was repeated until the difference between two successive weighing was less than 1 mg and the lowest weight recorded.

$$\% \text{ Moisture content} = \frac{100 (M_1 - M_2)}{M_1 - M}$$

Where; M₁ = Weight in gram of dish with material before drying; M₂

= Weight in gram of dish with the dried material, and M = Weight in gram of empty dish

Determination of water activity (a_w)

This was done using water activity meter (Buchi, Switzerland). About 10 g of each *Fura* and 10 mL of each *Nunu* sample was placed on the sample scale pan and readings recorded.

DNA extraction

Genomic DNA was extracted from the isolates using an extraction kit (Zymo Research, U.S.A.) following the procedure of the manufacturer. A 72- h tryptoy soya broth culture of the isolates was transferred in an Eppendorf tube and centrifuged for 5 minutes to pellet the cells. A 200 µL volume of nuclease free water was added to the cell pellet and vortexed for 1 min in a vortex mixer to wash the cells, it was then centrifuged for 1 min and the supernatant was decanted out. A 50 to 100 mg weight of the bacterial cells was resuspended in 200 µL of nuclease free water and transferred to a ZR Bashing Bead Lysis tube. Then, 750 µL of lysis solution was added to the tube. The tube was then vortexed in a vortex mixer vigorously for 5 to 10 min to lyse the cells. The ZR Bashing Bead Lysis tube was then centrifuged for 1 min at 10,000 × g. About 400 µL of supernatant was transferred to a Zymo-Spin IV Spin Filter in a collection tube and centrifuged at 7,000 × g for 1 min. To the filtrate in the collection tube, 1,200 µL of bacterial DNA Binding Buffer was added. About 800 µL of the mixture was transferred to a Zymo-Spin IIC column in a collection tube and centrifuged at 10,000 × g for 1 min. The flow through the collection tube was discarded and the process repeated. Then, 200 µL of DNA Pre-wash buffer was added to the Zymo-Spin IIC column in a new collection tube and centrifuged at 10,000 × g for 1 min. Next, 500 µL of bacterial DNA Wash Buffer was added to the Zymo-Spin IIC column and centrifuged at 10,000 × g for 1 min. The Zymo-Spin IIC column was finally transferred to a clean Eppendorf tube and 50 µL of DNA Elution Buffer was added directly into the column matrix and centrifuged at 10,000 × g for 30 s to elute the DNA.

PCR identification of *L. monocytogenes*

A 25 µL PCR supermix comprising 12.5 µL One Taq Quick-Load 2× Master Mix with Standard Buffer (New England Biolabs, U.S.A.), 0.5 µL of each primer (10 µM), 4.5 µL of nuclease free water and 5 µL of bacterial genomic DNA solution was subjected to thermocycling conditions, in a TECHNE thermocycler (Bibby Scientific, UK), according to the following schedule: Initial denaturation, 95°C for 5 min; 35 thermal cycles of 95°C, 30 s; 58°C, 30 s; 72°C, 45 s; final extension, 72°C for 5 min; and hold, 4°C. The PCR products were analysed on a 1.5% agarose gel containing ethidium bromide (10 mg/mL) and specific DNA bands were visualized using a bench top UV transilluminator (Upland, U.S.A.). Primers used for PCR are shown in Table 1. Identification of *L. monocytogenes* was by detection of the 370 and 456 bp amplicon of the *prs* and *hlyA* genes, respectively.

Serogrouping of *L. monocytogenes* and detection of virulence genes

Serogroups of the isolates were determined by PCR using specific primers (Table 1) for the three major serogroups (1/2a, 1/2b and 4b) associated with human listeriosis. The PCR conditions were: Initial denaturation, 94°C for 5 min; 35 thermal cycles of (94°C, 40 s; 53°C, 75 s; 72°C, 75 s); final extension, 72°C for 7 min; and hold,

Table 1. Nucleotide sequences of primers used in this study.

Primer name	Primer sequence (5'-3')	Product size (bp)	Primer target	References
<i>prs</i>	F: GCTGAAGAGATTGCGAAAGAAG R: CAAAGAAACCTTGGATTTGCGG	370	Genus <i>Listeria</i>	Doumith et al., 2004
<i>lmo0737</i>	F: AGGGCTTCAAGGACTTACCC R: ACGATTTCTGCTTGCCATTC	691	* <i>Lm</i> 1/2a	Doumith et al., 2004
<i>ORF 2819</i>	F: AGCAAAATGCCAAAACCTCGT R: CATCACTAAAGCCTCCCATTG	471	<i>Lm</i> 1/2b	Doumith et al., 2004
<i>ORF 2110</i>	F: AGTGGACAATTGATTGGTGAA R: CATCCATCCCTTACTTTGGAC	597	<i>Lm</i> 4b	Doumith et al., 2004
<i>plcA</i>	F:CTGCTTGAGCGTTCATGTCTCCATCCCCC R: CATGGGTTTCACTCTCCTTCTAC	1484	<i>plcA</i> gene	Momtaz & Yadollahi, 2013
<i>hlyA</i>	F: GCAGTTGCAAGCGCTTGGAGTGAA R: GCAACGTATCCTCCAGAGTGATCG	456	<i>hlyA</i> gene	Swetha et al., 2012
<i>iap</i>	F: ACAAGCTGCACCTGTTGCAG R: TGACAGCGTGTGTAGTAGCA	131	<i>iap</i> gene	Swetha et al., 2012

**Lm* = *L. monocytogenes*.

4°C. The isolates were also screened for the presence of virulence genes, *hlyA* and *iap* by PCR, under the same conditions described under DNA amplification above.

RESULTS AND DISCUSSION

Out of a total of 75 *Fura* and 75 *Nunu* samples examined in this study, 23 (30.7%) and 41 (54.7%) samples, respectively, yielded *Listeria* spp, based on growth on PALCAM *Listeria* agar, *Listeria* chromogenic agar and biochemical characterization. These values indicate that both the cereal and milk used in preparation of the RTE *Fura-de-Nunu*, were contaminated with *Listeria* spp. The occurrence recorded in this study are very much higher than the values of about 5 to 7% reported by some authors for raw milk and dairy products (Jamali et al., 2013; Marnissi et al., 2013; Mansouri-Najand et al., 2015; Shamloo et al., 2016), but lower than 78% reported from a study in south western Nigeria on an RTE soft cheese product (Oyinloye, 2016). Six presumptive *Listeria* species were identified from the samples. The distribution of the presumptive *Listeria* spp. in the *Fura* was as follows: *L. ivanovii* (36.7%) > *L. monocytogenes* (30.0%) > *L. seeligeri* (20.0%) > *L. grayi* (10.0%) and *L. welshimeri* (3.3%), while for *Nunu*, it was *L. ivanovii* (46.4%) > *L. monocytogenes* (26.8%) > *L. seeligeri* (16.1%) > *L. innocua* (5.4%) > *L. grayi* (3.6%) and *L. welshimeri* (1.8%). Another study in Nigeria had also reported these species to be the most predominant *Listeria* species in raw milk and attributed their presence

in milk to unhygienic milking practices (Yakubu et al., 2012). Among the *Listeria* species, *L. monocytogenes* is the most commonly associated with human listeriosis. Although the illness is relatively rare, it can have mortality rates as high as 30%, especially among vulnerable groups such as infants, pregnant women and the elderly (Mansouri-Najand et al., 2015). For this reason, most western countries have developed set-down policies and guidelines to monitor and control *L. monocytogenes* in foods. Unfortunately, the same cannot be said for Nigeria and some other African countries. The mean *Listeria* counts for the samples in this study were 7.7×10^4 CFU/g and 8.6×10^4 CFU/mL, for *Fura* and *Nunu*, respectively. Although these values represent total *Listeria* counts and not *L. monocytogenes* counts, they are still a cause for concern, considering that *L. monocytogenes* was the second most predominant species isolated from the food samples. According to international standards for acceptable levels of *L. monocytogenes* in foods, foods with a shelf life of less than five days or foods with the potential to support the growth of *L. monocytogenes* should not contain more than 100 CFU/g of food; and when the food is intended for infants, there should be complete absence of *L. monocytogenes* per 25 g of the food (EC 2073/2005; FSA UK, 2010; Health Canada, 2011; FOOD Standards Australia, 2014). The high frequency of *L. monocytogenes* in this study and the high total *Listeria* counts suggest a possibility that the *L. monocytogenes* counts in these products could well exceed the acceptable limit of 100 cfu/g of food. Moreover, as *Fura-de-Nunu* is a street-vended food,

Table 2. Intrinsic parameters of *Fura* and *Nunu* samples.

Sample	a_w	%LA	%NaCl	%Wc	pH
Fura	0.76±0.02	0.31±0.04	2.6±0.17	58.11±8.59	4.5±0.1
Nunu	0.94±0.01	1.17±0.02	3.3±0.12	91.13±1.87	3.6±0.1

Values represent means ± standard deviation of triplicate experiments; a_w = Water activity; LA = lactic acid; NaCl = Sodium chloride; Wc = Water content.

there is no specification for the groups of individuals that can consume the product. It is available to all groups of consumers including infants, pregnant women and the elderly; it is in fact considered a complete food, suitable for weaning babies and should therefore meet the standard of 'complete absence of *L. monocytogenes* / 25 g of food'. Other studies conducted in Nigeria have also reported high loads of *L. monocytogenes* from different foods, including RTE foods (Chukwu et al., 2006; Ikeh et al., 2010; Nwaiwu, 2015).

According to Food Safety guidelines, methods that have been recommended to control the growth of *Listeria* in foods including the use of antibacterial treatments, pasteurization, reduction of pH, refrigeration and consumption within safe periods (FSA UK, 2010; Health Canada, 2011; FOOD Standards Australia, 2014). However, for the RTE food studied here, none of these measures are usually applied. The products are made crudely by local Fulani women and then hawked in pans and containers on the streets, under conditions which would surely support growth of the *Listeria* organisms.

Evaluation of the physico-chemical properties of the samples showed that the pH and water activity (a_w) values of the *Fura* and *Nunu* samples were 4.5± 0.1, 0.76±0.0 and 3.6 ±0.1, 0.94±0.0, respectively (Table 2). Although these values appear to be within the safe ranges (pH < 4.4 and a_w <0.92), the lack of specified shelf life and the conditions under which the foods are vended still leave reasons for concern. According to the Canadian Bureau of Microbial Hazards policy on *L. monocytogenes* in RTE foods (2011), growth of *L. monocytogenes* is assumed to occur in RTE foods if the pH and a_w values fall outside the safe range, unless the RTE food processor is able to present data, to be reviewed by regulatory authorities which demonstrates that the growth of *L. monocytogenes* will not occur within the specified shelf life (Health Canada, 2011). Sadly, for the RTE *Fura de Nunu*, these criteria are neither considered nor met, before the street-hawking of the products.

Representative *L. monocytogenes* isolates (n=23) identified by phenotypic and biochemical methods were subjected to PCR, which has proved to be a very useful and rapid method for detection of *Listeria* (Mansouri-Najand et al., 2015). Screening of the presumptive *L. monocytogenes* isolates by PCR showed that only about 77% of the isolates could be confirmed as *L.*

monocytogenes, by detection of the 370 bp amplicon and 456 bp amplicon of the *prs* and *hlyA* genes, for identification of genus *Listeria* and *L. monocytogenes*, respectively (Figures 1 and 2). This result suggests that non-molecular methods of detection of these organisms may not be very specific and may lead to artificially high prevalence values. Thus, more specific molecular methods such as PCR must be used when evaluating *L. monocytogenes* contamination of food samples. The value of molecular methods of identification and typing has also been emphasized by other authors. For instance, it has been suggested that molecular methods are useful not just for accurate identification, epidemiological and trace-back investigations, but also for understanding the diversity and evolution of the organism (Doumith et al., 2004; Laksanalamai et al., 2014). It has also been suggested that although the classical biochemical methods of identification are standard, they can sometimes give false positives (Nwaiwu, 2015).

L. monocytogenes has 13 serotypes (1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4b, 4ab, 4c, 4d, 4e, and 7), but serogroups 1/2a, 1/2b and 4b are believed to be responsible for about 95% of human listeriosis (Kathariou, 2002; Jamali et al., 2013). Out of 24 *L. monocytogenes* (23 isolates and reference strain) screened by PCR-serogrouping in this study, only seven (including the reference strain) could be placed in any of the three serogroups tested for; and of these, only 1/2a and 4b were identified (Table 3). The 1/2a isolates were positive for *Imo0737* gene alone while the 4b isolates showed the presence of *ORF 2110*. Therefore, even though it has been suggested that variant strains of one serotype can sometimes acquire the gene clusters of another as in the reports of Laksanalamai et al., (2014), no such variants were detected in this study. Due to the epidemiological and clinical relevance of serotypes of the organism, serotyping is usually recommended for suspected *L. monocytogenes* isolates, particularly since the development of a PCR-based grouping scheme by Doumith et al. (2004). This molecular serogroup-related PCR typing has been endorsed by other researchers (Huang et al., 2011; Laksanalamai et al., 2014).

It has been suggested that pathogenic *L. monocytogenes* may be identified based on detection of some virulence markers, including internalins (*inIA*, *inIC*, *inIJ*), listeriolysin O (*hlyA*), actin polymerization protein

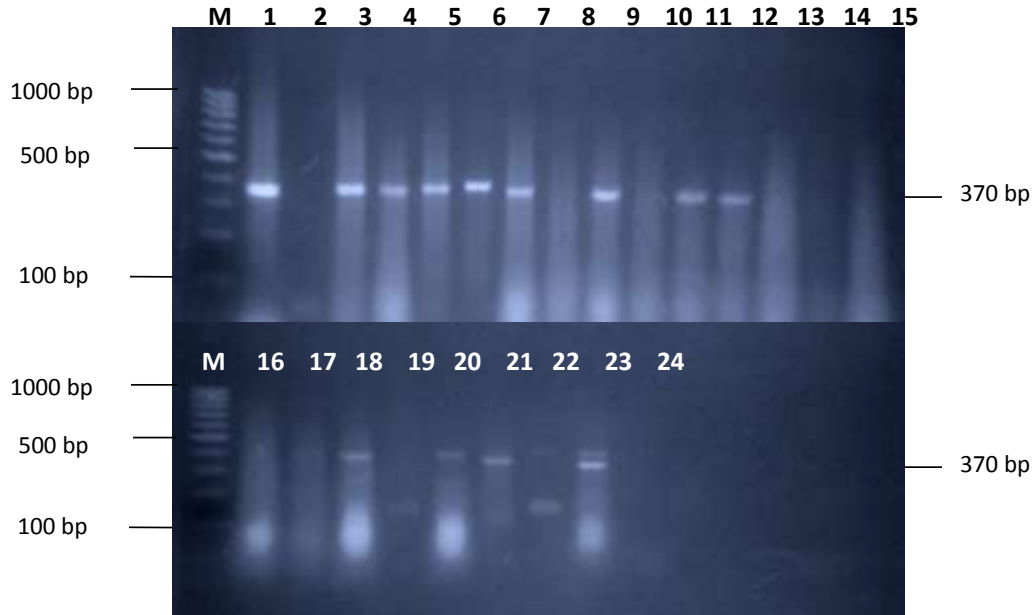


Figure 1. PCR detection of 370 bp amplicon of *prs* gene for identification of *Listeria* species. Lane M, 100 bp DNA ladder; lane 1, positive control (*L. monocytogenes* ATCC13932); lanes 2-23, presumptive *L. monocytogenes* isolates; lane 24 negative control. Lanes 3, 4, 5, 6, 7, 9, 11, 12, 18, 20, 21 and 23 are positive.

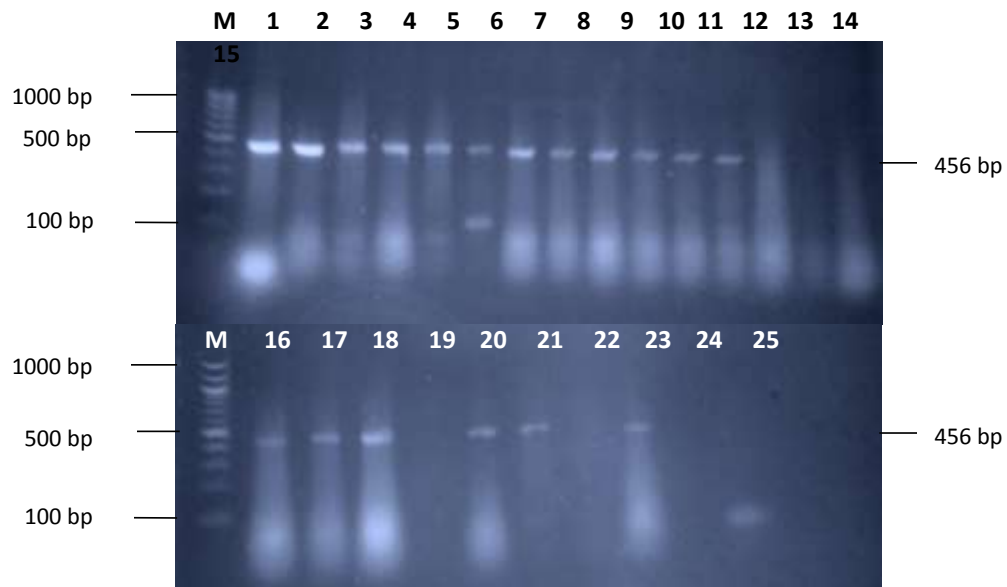


Figure 2. PCR detection of 456 bp amplicon of *hlyA* gene for identification of *L. monocytogenes*. Lane M, 100 bp DNA ladder; lane 1, positive control (*L. monocytogenes* ATCC13932); lanes 2-23, presumptive *L. monocytogenes* isolates; lane 25 negative control. Lanes 2-12, 16-18, 20, 21 and 23 are positive.

(*actA*), phosphatidyl-inositol-phospholipase C (*plcA*), invasive associated protein (*iap*) and virulence regulator (*prfA*), which are believed to be important in *L. monocytogenes* infection and pathogenesis (Liu et al.,

2007; Di Ciccio et al., 2012). PCR-based methods have also been used to identify pathogenic *L. monocytogenes* strains possessing these genes (Huang et al., 2011; Laksanalamai et al., 2014; Swetha et al., 2015). The

Table 3. Genetic characteristics of some *L. monocytogenes* isolates from *Fura* and *Nunu*.

Isolate ID	Source	PCR amplification reaction						PCR serogroup
		<i>prs</i>	<i>hlyA</i>	<i>iap</i>	<i>Lmo 0737</i>	<i>ORF 2819</i>	<i>ORF 2110</i>	
N21	Nunu	+	+	+	-	-	+	4b
N54	Nunu	+	+	+	-	-	+	4b
N69	Nunu	+	+	+	-	-	+	4b
N71	Nunu	+	+	+	+	-	-	1/2a
N72	Nunu	+	+	+	+	-	-	1/2a
F3	Fura	+	+	+	+	-	-	1/2a
TC	ATCC 13932	+	+	+	-	-	+	4b

isolates in this study were screened for the presence of two virulence genes: *hlyA* and *iap* by PCR and both genes were detected in some of the isolates (Table 3). Detection of these virulence genes suggests that the *L. monocytogenes* isolates from this study are pathogenic. Further studies will aim to carry out full genome sequencing on these isolates, in order to further characterize and document the strains. The isolation of virulent *L. monocytogenes* from the RTE product, *Fura-de-Nunu*, in this study is a significant cause for concern. *Fura de Nunu* is considered to be a complete food and is relatively cheap. Therefore, in a country with a large population of low-income earners, it comes highly recommended as a means of reducing malnutrition and is widely consumed across demographic groups, including pregnant women, infants and the elderly, who are among the groups most at risk for listeriosis. The results from this study highlight a need for the development and implementation of food safety policies and standards to guide the production and distribution of RTE foods in Nigeria. Producers and consumers of RTE products should also be made aware of the need to observe the highest possible standards of hygiene during production of RTE and street-vended foods to reduce the risk of listeriosis and other food-borne diseases.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

The authors would like to acknowledge Dr. Okorie-Kanu of the Department of Public Health and Preventive Medicine, Faculty of Veterinary Medicine, University of Nigeria, Nsukka, who graciously provided the *Listeria monocytogenes* reference strain.

REFERENCES

Abdellrazeq GS, Kamar AM, El-Houshy SM (2014). Molecular

- characterization of *Listeria species* isolated from frozen fish. Alexandria Journal of Veterinary Science, 40:1-15.
- Alsheikh ADI, Mohammed GE, Abdalla MA (2013). Isolation and identification of *Listeria monocytogenes* from retail broiler chicken ready-to-eat meat products in Sudan. International Journal of Animal and Veterinary Advances, 5:9-14.
- Barton BC, Jones TF, Vugia DJ, Long C, Marcus R, Smith K, Thomas S, Zansky S, Fullerton KE, Henao OL, Scallan E (2011). FoodNet Working G. Deaths associated with bacterial pathogens transmitted commonly through food. Foodborne Diseases Active Surveillance Network (FoodNet), 1996-2005. Journal of Infectious Diseases, 204:263-267.
- Chukwu OOC, Ogbonna CIC, Olabode AO, Chukwu AI, Onwuliri FC, Nwankiti OO (2006). *Listeria monocytogenes* in Nigeria processed meats and ready-to-eat dairy products. Nigeria Journal of Microbiology, 20:900-904.
- Chukwuma M (2009). Furors over animal milk products rage. World Journal of Microbiology and Biotechnology, 5:23-30.
- De Noordhout CM, Devleeschauwer B, Angulo FJ, Verbeke G, Haagsma J, Kirk M, Havelaar A, Speybroeck N (2014). The global burden of listeriosis: a systematic review and meta-analysis. Lancet Infectious Disease, 14:1073-1082.
- Di Ciccio P, Meloni D, Festino AR, Conter M, Zanardi E, Ghidini S, Vergara A, Mazzette R, Ianieri A, 2012. Longitudinal study on the sources of *Listeria monocytogenes* contamination in cold-smoked salmon and its processing environment in Italy. International Journal of Food and Microbiology, 158:79-84.
- Doumith M, Bucherrieser C, Glaser P, Jacquet C, Martin P (2004). Differentiation of the major *Listeria monocytogenes* serovars by multiplex PCR. Journal of Clinical Microbiology, 42:3819-3822.
- EC 2073/2005 (2005). Commission Regulation (EC) No 2073/2005 on microbiological criteria for foodstuffs. Official Journal of the European Union, L 338,1-26.
- Food Standards, Australia New Zealand (2014). Criteria for *Listeria monocytogenes* in ready-to-eat foods.
- Food Standards Agency, European Commission (2006). General guidance for Food Business Operators. EC Regulation No 2073/2005 on microbiological criteria for foodstuffs.
- Food Standards Agency, United Kingdom (2010). The food standard agency's *Listeria* risk management programme (LRMP) 2010-2015.
- Gillespie IA, Mook P, Little CL, Grant KA, Mclauchlin J (2010). Human Listeriosis in England, 2001-2007: association with neighbourhood deprivation. Eurosurveillance. 15 (27): pii=19609.
- Guillet C, Join-Lambert O, Le Monnier A, Leclercq A, Mechaï F, Mamzer-Bruneel MF, Bielecka MK, Scorti M, Disson O, Berche P, Vazquez-Boland J, Lortholary O, Lecuit M (2010). Human listeriosis caused by *Listeria ivanovii*. Emerging Infectious Diseases, 16:136-138.
- Health Canada (Bureau of Microbial Hazards Food Directorate, Canada) (2011). Policy on *Listeria monocytogenes* in Ready-to-Eat Foods.
- Huang B, Fang N, Dimovski K, Wang X, Hogg G, Bates J (2011). Observation of a new pattern in serogroup-related PCR typing of *Listeria monocytogenes* 4b isolates. Journal of Clinical Microbiology,

- 49:426-429.
- Ikeh MAC, Obi SKC, Ezeasor DN, Ezeonu IM, Moneke AN (2010). Incidence and pathogenicity profile of *Listeria* spp. isolated from food and environmental samples in Nsukka, Nigeria. *Africa Journal of Biotechnology*, 9:4776-4782.
- International Organization for Standardization (2004). Modification of the isolation media and hemolysis test and inclusion of precision data. ISO 11290-1:1996/And/1:2004. International Organization for Standardization, Geneva, Switzerland.
- Jamali H, Radmehr B, Thong KL (2013). Prevalence, characterisation, and antimicrobial resistance of *Listeria* species and *Listeria monocytogenes* isolates from raw milk in farm bulk tanks. *Food Content*. 34:121-125.
- Jideani VA, Nkama I, Agbo ED, Jideani IA (2001). Survey of Fura production in some northern states of Nigeria. *Plant Food and Human Nutrition*, 56:23-26.
- Kathariou S (2002). *Listeria monocytogenes* virulence and pathogenicity, a food safety perspective. *Journal of Food Protection*. 65:1811-1829.
- Kordylasi JM (1990). Processing and preservation of tropical and sub-tropical foods, Macmillan, London, 112-114.
- Laksanalamai P, Huang B, Sabo J, Burall LS, Zhao S, Bates J, Datta AR (2014). Genomic characterization of novel *Listeria monocytogenes* serotype 4b variant strains. *PLoS ONE* 9, e89024. doi:10.1371/journal.pone.0089024
- Liu D, Lawrence ML, Austin FW, Ainsworth AJ (2007). A multiplex PCR for species-and virulence-specific determination of *Listeria monocytogenes*. *Journal of Microbiology, Methods* 71:133-140.
- Majjala R, Lyytikäinen O, Johansson T, Autio T, Aalto T, Haavisto L, Honkanen-Buzalski T (2001). Exposure of *Listeria monocytogenes* within an epidemic caused by butter in Finland. *International Journal of Food Microbiology*, 70:97-109.
- Mansouri-Najand L, Kianpour M, Sami M, Jajarmi M (2015). Prevalence of *Listeria monocytogenes* in raw milk in Kerman, Iran. *Journal of Veterinary Research, Forum*, 6:223-226.
- Marnissi BE, Bennani L, Cohen N, Lalami AEO, Belkhou R (2013). Presence of *Listeria monocytogenes* in raw milk and traditional dairy products marketed in the north-central region of Morocco. *Africa Journal and Food Science*, 7:87-91.
- Momtaz H, Yadollahi S (2013). Molecular characterization of *Listeria monocytogenes* isolated from fresh seafood samples in Iran. *Diagnostic Pathology*, 8:149.
- Nwaiwu O (2015). An overview of *Listeria* species in Nigeria. *International Food Research Journal*, 22:455-464.
- Okeke KS, Abdullahi IO, Makun HA (2014). Microbiological Quality of Dairy Cattle Products. *British Microbiology Research Journal*, 4:1409-1417.
- Ooi ST, Lorber B (2005). Gastroenteritis due to *Listeria monocytogenes*. *Clinical Infectious Diseases*, 40:1327-1332.
- Oyinloye JMA (2016). Detection and Molecular Characterization of *Listeria species* in 'Wara', A West African Local Cheese Sold in Ekiti State. *International Journal of Current Microbiology and Applied Sciences*, 5:941-948.
- Public Health England (2014). Detection and enumeration of *Listeria monocytogenes* and other *Listeria* species. Microbiology services. Food, water and environmental standard method FNES22 (F19); version 2.
- Shamloo E, Jalali M, Mirlahi M, Madani G, Metcalf D, Merasi MR (2014). Prevalence of *Listeria* species in raw milk and traditional dairy products in Isfahan, Iran. *International Journal of Environmental Health Engineering*, 3:1-5.
- Swetha CS, Rao TM, Krishnaiah N, Vijaya KA (2012). Detection of *Listeria monocytogenes* in fish samples by PCR assay. *Annals of Biological Research*, 3:1880-1884.
- Weller D, Andrus A, Wiedmann M, den Bakker HC (2015). *Listeria booriae* sp. nov. and *Listeria newyorkensis* sp. nov., from food processing environments in the USA. *International Journal of Systematic and Evolutionary Microbiology*, 65:286-292.
- Yadav MM, Roy A, Bhandari B, Josh C (2010). Pheno-genotypic characterization of *Listeria monocytogenes* from bovine clinical mastitis. *Buffalo Bulletin*, 29:29-38.
- Yakubu Y, Salihu MD, Faleke OO, Abubakar MB, Junaidu AU, Magaji AA, Gulumbe ML, Aliyu RM (2012). Prevalence and antibiotic susceptibility of *Listeria monocytogenes* in raw milk from cattle herds within Sokoto metropolis, Nigeria. *Sokoto Journal of Veterinary Science*, 10:13-17.
- Yang H, Qu L, Wimbrow AN, Jiang X, Sun Y (2007). Rapid detection of *Listeria monocytogenes* by nanoparticle-based immune-magnetic separation and real-time PCR. *International Journal of Food Microbiology*, 118:132-138.

Full Length Research Paper

Emphasis on functional properties of cocoa-specific acidifying lactic acid bacteria for cocoa beans fermentation improvement

Evelyne Cho Adiko, Ginette Gladys Doué*, Lessoy Thierry Zoué and Sébastien Lamine Niamké

Laboratory of Biotechnology, UFR Biosciences, Felix Houphouet-Boigny University, 22 BP 582 Abidjan 22, Ivory Coast.

Received 13 March, 2018; Accepted 7 May, 2018

Lactic acid bacteria (LAB) strains isolated from six main Ivorian cocoa producer regions were investigated based on their biochemical properties in order to select the best one as potential starter. Three main technological and useful properties for good cocoa beans fermentation were monitored among the 568 isolated LAB strains. Thus, between the 408 cocoa-specific acidifying LAB strains identified, 05.88% (24 isolates) of them were able to maintain this activity in pH and temperature conditions as same as fermentation heap with an acidifying rate ranged within 0.03 ± 0.00 to $1.19\pm 0.070\%$. All these 24 cocoa-specific acidifying LAB strains displayed the ability to degrade the citrate while six (25%) of them were able to produce acetoin. These six LAB strains namely T₁Gb₈, T₆C₅, T₆C₁₂, T₇C₈, T₉C₉ and T₁₁C₅ were identified as *Lactobacillus plantarum*. They were the best able to continuously produce lactic acid in the required standards. Firstly under varying condition of temperature (30 à 45°C) and pH (3 à 6) with sharp acid (acetic, lactic and citric acids) influence. Secondly beyond 8% of ethanol content. The highlighted properties in this study and the performance of these six strains may make them best candidate as starters for cocoa fermentation control.

Key words: Lactic acid bacteria, cocoa-specific acidifying, technological properties, starter, Ivorian cocoa, fermentation.

INTRODUCTION

Cocoa beans constitute a basic export product for many tropical countries in America (Central and South), Asia (South and Southeast) and mainly West Africa. Export of raw cocoa beans is of great economic importance for

West African countries which supply two thirds of the world's cocoa crop. Côte d'Ivoire is the world's leading producer of cocoa beans since 1978 (Deheuvels, 2003). Today, this country is highly dependent on this crop

*Corresponding author. E-mail: gladysdoue@yahoo.com.

which accounts for 50% of national export income and 22% of the Gross Domestic Product (ICCO, 2016). Cocoa beans are an important source of cocoa powder and the principal raw material of chocolate. The sensory and physical properties of West African cocoa make it a much sought-after product, although overall production quality seems to be declining (Afoakwa et al., 2013). Unfortunately, ensuring the marketable quality of cocoa beans remains difficult due to variability in the organoleptic quality of this product. This inconsistency in cocoa quality is mainly linked to post-harvest processing including fermentation, drying and roasting of the crude and fresh cocoa beans. Fermentation is considered as the most important processing step influencing the quality of cocoa beans. Indeed, during this process, there is initiation of flavor precursors, color development and a significant reduction in bitterness and astringency of the beans (Afoakwa et al., 2013). These changes involve the action of diverse microorganisms naturally found in the cocoa pulp. The succession of these microbial groups has been clearly established and lactic acid bacteria (LAB) population is considered as the second microbial group growing in mucilaginous pulp of cocoa beans after yeast population (Crafack et al., 2013). This micro-biota group is mainly dominating until 24 to 48 h of fermentation (Kouame et al., 2015). The most important roles of LAB are the degradation of the citric acid in the pulp, leading to increase in pH, the production of ethanol and both volatile and non-volatile organic acids such as acetic and lactic acids (Penia et al. 2013). Organic acid will diffuse into the beans cotyledon and induce the enzymatic reactions to form precursor of flavor, aroma and color (Afoakwa et al., 2013). The diffusion of certain amount of acid into the bean during fermentation is essential for the development of flavor precursors. Indeed, the presence of excessive acidity usually correlates with poor development of chocolate flavor (Serra et al., 1997). Thus, acidity is a critical factor in defining the quality of cocoa used in chocolate manufacturing. The control of cocoa fermentation process is very challenging for farmers in cocoa producing countries because cocoa fermentation has always been a natural process which sometimes results in poorly fermented cocoa. Therefore, studies suggest the use of starter microbial culture as the best approach to improve the fermentation process. In this perspective, LAB strains as monoculture or co-culture would be essential component of starter culture aimed at the control of cocoa beans fermentation process. Studies conducted by different authors, such as Papalexandratou et al. (2013), Penia et al. (2013) etc., on cocoa fermentation showed that the maximum rate of lactic acid produced ranged between 0.5 and 1.2%. As excessive acidity, mainly lactic acid, produces unsuitable raw cocoa for chocolate making, it appears more judicious to isolate, screen and characterize LAB strains with cocoa-specific acidifying potential included in this

limit of 1.2%. These strains may positively and durably influence the quality of a controlled cocoa fermentation especially if they provide useful additional properties such as citrate degradation and acetoin production (Rodriguez-Campos et al., 2012). Information on cocoa-specific acidifying LAB strains with adequate lactic acidifying potential, citrate degradation and acetoin production may be a starting point for the development of starter culture. Thus, the aim of the research was to select strains of LAB that best meet these different criteria in order to optimize and standardize the cocoa fermentation process.

MATERIALS AND METHODS

Fermentation condition

Cocoa pods were harvested on farms from six cocoa producing regions of Ivory Coast: Cavally (6° 25' 0" North -7° 30' 0" West), Gkôklè (4° 57' 04" north 6° 05' 19" West), Gôh (6° 15' 0" North 5° 55' 0" West), Haut-Sassandra (7° 0' 0" North -6° 34' 59" West), San-Pedro (4° 44' 54" North -6° 38' 10" West) and Tonkpi (7° 24' 45" North 7° 33' 14" West). Spontaneous cocoa bean fermentation was performed using traditional conditions, with temperature and humidity ranging respectively, from 28 to 30°C, and 60 to 65% for 6 days. The fermenting mass (50 kg) set on banana leaves and covered with banana leaves were constituted of mixed genotypes (Forastero, Trinitario and Criollo cultivars).

Sampling

The fermenting heap was mixed and 100 g beans were collected, at the beginning and each 12 h of fermentation, in sterile Stomacher bag. A total, 13 samples of each fermenting cocoa according to the locality were collected for physico-chemical and microbial analysis.

Isolation of bacterial strains

Isolation of LAB was performed according to the standard method described by Pereira et al. (2012). An amount of 25 g of fermenting cocoa beans was homogenized in 225 mL sterile peptone water in a Stomacher bag (Seward, Worthington, United Kingdom) for 5 min at room temperature. After appropriate dilution of samples in sterile saline solution, 0.1 mL from each dilution was inoculated onto duplicate plates of MRS agar (Oxoid) supplemented with 50 µg/mL of nystatin to inhibit the fungal growth. Plates were incubated at 30°C for 48 to 72 h, under anaerobic conditions.

Preliminary biochemical test of lactic acid bacteria

LAB isolates, identified using conventional colonial morphology, gram staining and biochemical reactions (oxidase and catalase tests), were stored in cryotubes at -20°C in MRS broth supplemented with 20% glycerol for further investigations.

Screening of cocoa-specific acidifying LAB strains

Cocoa-specific acidifying LAB strains were screening based on their acidification capacity at 30°C according to the protocol of Guha et al. (2013). 100 µL of a preculture recording an optical

density (OD) of 1 at 600 nm against a control (MRS broth) were used to inoculate 5 mL of MRS broth. The seeded broths were incubated at 30°C in a water bath (Julabo TWB 12) for 48 h. Then, fermented broths were centrifuged at 4500 trs/min for 10 min to pellet out the bacterial growth. Supernatant was used to estimate lactic acid content by titrimetric method with 0.1 NaOH. The percentage of lactic acid was calculated using the formula in equation 1. Each treatment was tested in triplicate.

$$\% \text{ Lactic acid} = V(\text{NaOH}) \times N \times 0,09 \times 100/V(\text{test}) \quad (1)$$

V(NaOH) : Volume of NaOH solution used for titration

N : Normality of the NaOH solution (0.1)

V (test): Sample Volume

Analysis of additional properties of cocoa-specific acidifying lactic acid bacteria isolates

Screening of LAB strains for citrate degradation

The ability of lactic acid bacteria to degrade citrate was investigated using the method described by Kempler and McKay (1980). A basal medium containing 1% milk powder, 0.25% casein peptone, 0.5% glucose and 1.5% agar was first prepared and then 1L of the medium, was supplemented with 10 mL of potassium ferricyanide (10%) solution and 10mL of mixed iron citrate and sodium citrate (2.5%) solution. The medium was inoculated with pure 24 h preculture of LAB strains and incubated in an anaerobic jar at 30 °C for 48 to 72 h. The citrate metabolism was assessed by the formation of a blue complex surrounding the colonies.

Screening of LAB strains for acetoin production

The production of acetoin was investigated using the method described by King (1948). Acetoin production from citrate was checked in the basal liquid medium. Clark and Lubs medium was seeded with pure 24 h preculture of LAB strains and incubated at 30°C for 48 h. After incubation time, acetoin production was revealed in 1mL of cell free supernatant by adding two drops of an alcoholic solution of alpha-naphthol (6%) and two drops of sodium hydroxide (16%) solution. The presence of acetoin was assessed by appearance of red color.

Effect of environmental stress on lactic acid production by cocoa-specific acidifying LAB strains

Influence of temperature and pH

The influence of temperature and pH on the acidification capacity of cocoa-specific acidifying lactic acid bacteria strains was evaluated by Guha et al. (2013) method. Seeded broths were incubated at 30, 35, 40, 45 and 50 °C to assess the influence of temperature and at pH 3; 4; 5 and 6 for pH influence, in a water bath (Julabo TWB 12) for 48 h.

Influence of alcohol and organic acids on lactic acid production by cocoa-specific acidifying LAB strains

To analyze the acidification potential of cocoa-specific LAB strains in alcoholic and acidic conditions, a MRS liquid medium was prepared. After autoclaving, this medium was cooled and maintained in liquid state at 45 °C and supplemented with alcohol

(2, 4, 6 and 8%), acetic and lactic acid (0.2; 0.4; 0.6; 0.8; 1; 1.2%), citric acid (0.5; 1; 1.5; 2; 2.5 and 3%). The standard inoculum was prepared as follow: a 18 h preculture was suspended in a saline solution to obtain an absorbance OD 1 at 600 nm. 100 µL were used to inoculate 5 mL of MRS broth. The seeded broths were incubated at 30°C in a water bath for 48 h. After incubation time, the amount of total acid produced by each strain was determined by titration as previously described.

Molecular characterization of lactic acid bacteria starters

16S rRNA gene PCR and sequence analysis

To perform PCR reactions, The isolates of LAB strains were grown for 24 h at 30°C on agar plates. A loopful of pure culture was suspended in 100 µL of sterile distilled water for colony PCR.

PCR amplification of ribosomal 16S gene

The hypervariable regions (V1, V2 and V6) of the different LAB strains were amplified by using universal primers F27 (5'-AGAGTTTGTATCCTGGCTCAG-3') and R520 (5'-ACCGCGGCTGCTGGC-3') (Ouattara et al., 2011) for colony PCR. Each mixture (final volume 50 µL) contained about 1 µL of sample 1.25 U of Taq DNA polymerase (Biolabs, Lyon France), 5 µL of 10x Taq Buffer (10 mM TrisHCl, 50 mM KCl, 1,5 mM MgCl₂), 1 µL of 10mM dNTP, 2 µL of each primer (10 µM) (Eurofins, Genomics, Germany) supplemented by sterile water Milli-Q. PCR amplification was carried out in a thermocycler (Sensoquest Labcycler) as described previously (Ouattara et al., 2011). After an initial denaturation at 95 °C for 4 min, reactions were run for 35 cycles. Each cycle comprising denaturation at 95 °C for 1 min, annealing at 56 °C for 1 min, extension at 72 °C for 1min; and final extension at 72 °C for 10 min.

Gel electrophoresis

After migration of the products realised at 70 V for 2 h in a tank, amplicons were revealed by incubating on 0.8% agarose gel electrophoresis in a 1x phosphate TAE buffer containing bromide of ethidium. The molecular weight of the products was estimated in reference to a molecular weight marker (Biolabs, France) of 500 bp.

Sequencing and analysis of hypervariable sequence of 16S RNA gene amplified

The PCR products were purified using the nucleospinR Gel and PCR Clean-up kit (MachereyNagel, Germany). Then, they were sequenced by MWG Eurofins using the primer F27. The basic local alignment search tool (BLAST, blast N) from the NCBI database site (blast.ncbi.nlm.nih.gov/ BLAST/) was used to find the closest sequences relative to the amplified 16S RNA genes in order to identify our LAB strains.

RESULTS AND DISCUSSION

Acidification capacity of LAB strains isolates

A total of 568 strains characterized as gram positive, oxydase negative and catalase negative identified as

Table 1. Distribution of LAB strains isolates based on their acidifying capacity.

Regions	S.I.	A.C. of total LAB strains isolates			C.S.A.LabStrains (A.R.≤1.2%)
		Low acidity LAB strains A.R.< 0.45%	Medium acidity LAB strains 0.45<A.R.≤1.35%	High acidity LAB strains A.R.≥ 1.35%	
Cavally	104 (18.31)	00 (00)	40 (12.23)	64 (46.71)	036 (08.82)
Gboklè	087 (15.33)	05 (04.81)	78 (23.85)	04 (02.92)	078 (19.12)
Goh	048 (08.45)	15 (14.42)	15 (04.59)	18 (13.14)	028 (06.86)
H. S.	109 (19.19)	25 (24.04)	84 (25.69)	00 (0.00)	107 (26.23)
S. P	122 (21.47)	59 (56.73)	63 (19.27)	00 (0.00)	121 (29.66)
Tonkpi	098 (17.25)	00 (00)	47 (14.37)	51 (37.23)	038 (09.31)
Total Strains	568 (100)	104(8.31)	327 (57.57)	137 (24.12)	408 (71.83)

Note: With regard to the acidity property, C.S.A.LAB strains (A.R.≤1.2%): correspond to the Low and Medium acidity LAB strains with acidifying potential ≤1.2% ; H. S.: Haut sassandra; S. P.: San pédro; S.I.: Strains Isolates; A.R.: acidification rate. A.C. of LAB strains isolates: Acidifying capacity of LAB strains isolates; C.S.A.LAB strains: Cocoa-specific acidifying LAB strains.

LAB were isolated from six Ivorian regions. Thus, these LAB strains were analysed for their capacity to acidify the fermentative medium. The results showed that the number of isolated LAB strains have unequal distribution depending on the region. San Pédro recording the highest (21.47%) number of isolates (Table 1) unlike Gôh, which has the lowest number (08.45%) of strains. This variability indicated the influence of the local geographical area on the composition of the microflora involved in cocoa fermentation (Schwan and Wheals, 2004). Therefore, this factor could impact on the distribution of their different activities. One of the important activities attributed to LAB strains is to acidify the fermentative medium. As this property is susceptible to play a key role in the fermentative process (Afoakwa et al., 2013), it appears important to screen them based on this ability. Most importantly, this type of metabolic pathway should be determined to identify potential starters among these isolates.

All the 568 isolated LAB microflora which were screened for their acidification capacity showed naturally acidification capacity with different levels of acid production (Table 1). Indeed, this natural capacity for acidification of the fermentative medium by LAB is related to the fact that they are able to exhibit an enormous capacity to degrade different carbohydrates and related compounds. Generally, the predominant end product is lactic acid which is the main metabolite of two broad metabolic categories of LAB. The first metabolic category is represented by the homofermentative LAB. They convert glucose into lactic acid more than 90% by the Embden-Meyerhof (E-M) pathway. The second metabolic group is represented by the heterofermentative LAB producing about 50% of lactic acid as the principle by-product of sugar fermentations (Raimbault 1995).

It should be noted that a acidification scale has been established based on the acidification capacity of LAB from different fermentation medium such as milk, cheese,

wine, fish, meat, sausages, butter and cream (Behannis and Kayanush, 2012; Joko et al., 2014). The recorded lactic acid values (around twenty) ranged between 0.05% and 1.6% and allowed to identify three types of LAB strains according to their capacity of acidification: LAB strains with low acidity capacity (acidification rate (AR) < 0.45%); LAB strains with medium acidity capacity (0.45 < AR ≤1.35%) and those with high acidity capacity (AR ≥ 1.35%).

The results obtained are depicted in Table 1. Based on this classification, three types of LAB strains were observed in the different regions except in Cavally and Tonkpi with respect to the low lactic acid producing strains and the regions of Haut Sassandra and San Pédro with respect to the high producers. In addition, medium acidifying LAB strains dominated with 57.57% compared to 24.12% for high acidifying LAB strains and only 18.31% for low acidifying LAB strains. This diversity in acidifying capacity of LAB strains suggesting a difference in acidification potential and therefore efficiency in acidification by these strains. This could be the main determining factor in the variability of cocoa bean quality as cocoa beans acidification often has negative impact on the final quality of the market cocoa.

Therefore, the cocoa market could ultimately be related to cocoa bean quality which is dependant on selective LAB strains with satisfactory level of acidification. This approach could make it possible to ensure the control of the acid level of marketable beans and thus the control of their quality. With regard to lactic acid, the satisfactory level of acidification can only be obtained with LAB strains that do not have high acidification capacity. Indeed, the low volatility of this acid promotes its accumulation in beans. If this acid is produced in large quantities, it can hardly be eliminated. This will irretrievably lead to acidic beans and therefore of poor quality. Thereby, LAB strains with low and medium acidification capacity (431) retained our attention,

Table 2. Influence of temperature on lactic acid production by Cocoa-specific acidifying LAB strains per region.

Regions	Number of LAB strains with acidifying potential $\leq 1.2\%$ at			
	30°C (%)	35°C (%)	40°C (%)	45°C (%)
Cavally	036 (08.82)	023(09.02)	022(09.73)	17 (16.35)
Gbôklè	078 (19.12)	062(24.31)	058(25.66)	34 (32.70)
Gôh	028 (06.86)	015(05.88)	011(04.87)	06 (05.77)
H.S.	107 (26.23)	081(31.76)	072(31.86)	25 (24.04)
San-Pedro	121 (29.66)	058(22.75)	050(22.12)	16 (15.38)
Tonkpi	038(09.31)	016(06.27)	013(05.75)	06 (05.77)
Total	408 (100)	255 (100)	226 (100)	104 (100)

approximately two-thirds of LAB strains isolated.

Then, a second level of selection has been established to better refine our selection. This selection takes into account the maximum rate of lactic acid produced during the fermentation of cocoa. Studies conducted by Afokawa (2013), Kouame et al. (2015) and Papalexandratou et al. (2013; 2015) on cocoa fermentations, around the world, lead to the identification of a maximum rate of lactic acid produced of 1.2%. Thus, in order to mimic the natural conditions of fermentation, it is important that the selected LAB strains do not produce lactic acid beyond this limit (1.2%). The results of this selection are shown in Table 1. Among the 568 strains of LAB previously isolated, approximately two-thirds (71.83%) produced an amount of lactic acid that not exceed 1.2%. This total of 408 LAB strains applies to all the strains with low acidifying capacity (104) and part of the medium acidifying strains (304 of 327). These strains are considered to be "Cocoa-specific acidifying LAB strains". As high acidity, mainly lactic acid, in cocoa beans produces unsuitable raw cocoa, these 408 Cocoa-specific acidifying LAB strains appear to be the best candidates as potential starter culture to control cocoa bean fermentation.

Influence of temperature and pH on lactic acid production by Cocoa-specific acidifying LAB strains

The pH of cocoa beans during fermentation is critical with regard to the biochemical reactions that take place in the beans (Afokawa et al., 2013). The ability of the 408 Cocoa-specific acidifying LAB strains to maintain their acidifying potential $\leq 1.2\%$ at temperatures up to 45°C is depicted in Table 2. Only 104 strains maintained their acidifying potential under 45°C of temperature among the 408 strains previously selected.

Cocoa fermentation is generally characterized by high temperature up to 45°C occurring between 48 and 72 h of fermentation (Ho et al., 2014). In view of these results, we can assess that the thermo-tolerance exhibited by these 104 heat-resistant strains should allow them to

maintain a satisfactory level of lactic acid production under thermic stress conditions during cocoa fermentation.

On the other hand, the effect of pH variations on these 104 heat-resistant strains, showed that only 24 of them were able to continuously maintain their acid production for pH variations ranging from 3 to 6 (data not showed). These pH values are those prevailing in cocoa beans fermentation within 24 to 72 h of fermentation (Kouame et al., 2015). Table 3 highlights the amounts of lactic acid produced by these 24 cocoa-specific acidifying LAB strains under pH fluctuation.

Regarding the rate of lactic acid produced by these Cocoa-specific acidifying LAB strains, values are ranged from a minimum of 0.03 to a maximum of 1.19 for pH values between 3 and 6 (Table 3). These findings pointed out the fact that these Cocoa-specific acidifying LAB strains did not have the same capacity of adaptation to temperature and pH fluctuations of the fermentative heaps even if they are able to maintain their lactic acid production in the required standards.

Additional activity of isolated Cocoa-specific acidifying LAB strains: citrate degradation and acetoin production

Citrate degradation and acetoin production are important and relevant properties desirable for a good cocoa fermentation (Schwan and Wheals, 2004). Thus, biochemical characterization of the 24 Cocoa-specific acidifying LAB strains showed that they exhibited the property to break down citric acid. Additionally, only six (25%) of them displayed both the ability to degrade citrate and to produce acetoin.

The screening of citrate consumers among these cocoa-specific acidifying LAB strains indicates that all of them are able to breakdown citrate. Citrate metabolism constitutes, an important and particular property, since LAB strains are not usually able to utilize citric acid as carbon source (Kouame et al., 2015). As citrate metabolism is an essential factor for modulating the pH

Table 3. Amount of lactic acid produced by cocoa-specific acidifying LAB strains under pH variation per region.

Regions	Number of LAB strains isolates	Acidifying potential of cocoa-specific LAB (%) at			
		pH ₃	pH ₄	pH ₅	pH ₆
Gbôklè	04	0.03±0.014-0.30±0.057	0.04±0.00-0.12±0.02	0.50±0.071-0.80±0.226	1.06±0.057-1.19±0.070
H.S.	03	0.63±0.00-0.86±0.028	0.87±0.01- 1.04±0.00	1.02±0.042-1.10±0.170	1.16±0.030-1.12±0.056
San-Pédro	02	0.65±0.005-0.73±0.006	0.90±0.016-1.09±0.025	1.11±0.028- 1.10±0.150	1.14±0.064-1.16±0.046
Gôh	01	0.05±0.00	0.10±0.00	0.15±0.00	0.16±0.05
Cavally	10	0.08±0.00-0.67±0.002	0.10±0.00- 0.98±0.013	0.20±0.05-1.02±0.06	0.99±0.00-1.18±0.015
Tonkpi	04	0.03±0.00-0.20±0.001	0.10±0.00 0.85±0.113	0.50±0.00-1.06±0.06	0.91±0.001-1.18±0.008
Total	24	0.03±0.00-0.86±0.028	0.04±0.00 -1.09±0.025	0.15±0.00 -1.06±0.06	0.16±0.005 -1.19±0.070

H.S: Haut Sassandra.

and promoting the growth of desirable bacterial flora in fermenting cocoa (Schwan and Wheals, 2004) all these 24 LAB strains should be responsible for the raising of the pH recorded during cocoa beans fermentation.

In addition, acetoin production shows that these six cocoa-specific acidifying LAB strains (T₁Gb₈, T₆C₅, T₆C₁₂, T₇C₈, T₉C₉ and T₁₁C₅) were able to metabolized citric acid via pathways pII producing acetoin from citrate. This ketone carbonylic compound (such as acetoin) is crucial for the development of good cocoa flavor. Indeed, Rodriguez-Campos et al. (2012) indicated that acetoin appears to be a precursor of tetramethylpyrazine, an important odor-active component of cocoa flavor. Therefore, these six LAB strains are of interest for the fermentation process as potential starter.

16S rRNA gene PCR and identification of isolates

Amplification of hypervariable regions (V1, V2 and V6) of the 16S gene of six potential starters LAB

by colony PCR yielded a DNA fragment with respect to the 500bp (Figure 1). Analysis of these DNA fragments in the NCBI data bank led to the identification of these six LAB (T₁Gb₈, T₆C₅, T₆C₁₂, T₇C₈, T₉C₉ and T₁₁C₅) isolates as belonging to *Lactobacillus plantarum* with 99% of similarity. *L. plantarum* has been isolated from many cocoa fermentations around the world (Camu et al., 2007; Pereira et al., 2012). This observation indicates that, these strains play key roles in cocoa bean fermentation. *L. plantarum* species has also been used as starter for cocoa fermentation (Joko et al., 2014; Penia et al., 2013) and are widely used in food industries to make other fermented foods (Joko et al., 2014).

Influence of additional stress on lactic acid production by isolated Cocoa-specific acidifying LAB strains.

In addition to pH and temperature, other factors such as acids (acetic, lactic and citric) and alcohols (ethanol) are key parameters that may strongly influence the performance of the LAB

strains in lactic acid production. Thus, the six selected LAB strains (T₁Gb₈, T₆C₅, T₆C₁₂, T₇C₈, T₉C₉ and T₁₁C₅) and identified as *Lactobacillus plantarum* were tested to assess the influence of these key parameters on Their ability to maintain lactic acid production.

Effect of acids (acetic and lactic acids) on lactic acid production

In acid conditions induced by lactic, acetic and citric acids, the six selected LAB strains proved to be able to maintain their potential of lactic acid production despite the observed decline. Lactic acid production occurred in medium containing lactic and acetic acid in the same range 0-1.4% for the six strains belonging to *Lactobacillus plantarum* (Figure 2 (A and B)) while this acid production continues up to 1.6% of acetic acid in the medium. The influence of citric acid on lactic acid production was lesser than the other tested acids (Figure 2C). Lactic acid production was slowly decreased with increasing of citric acid concentrations in a range of 0-3%. All *Lactobacillus*

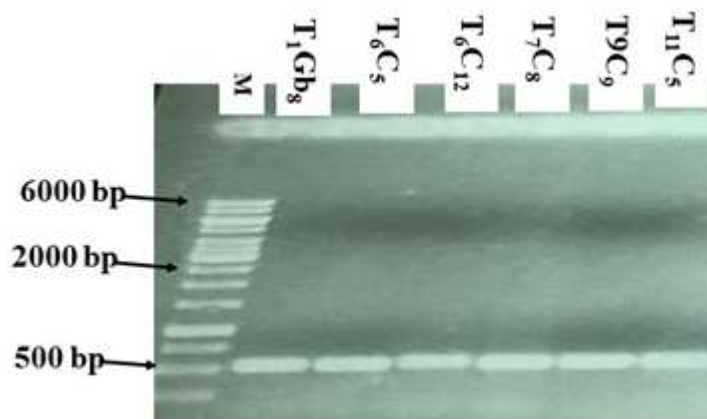


Figure 1. Electrophoretic profile of the PCR products of hypervariable regions of the 16S rRNA gene from the six LAB potential starters. M: molecular weight marker (ladders).

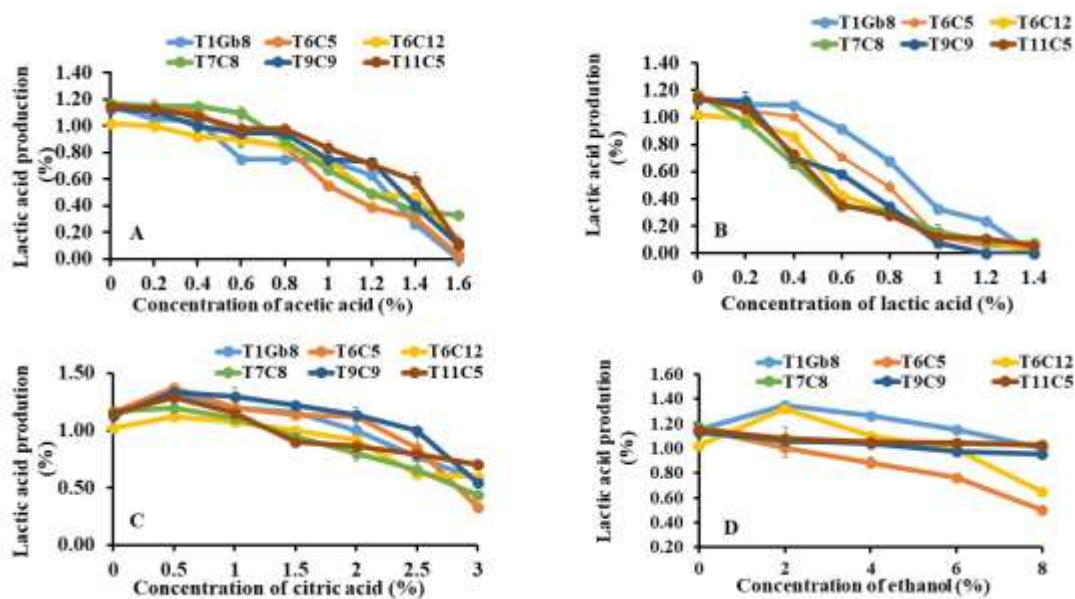


Figure 2. Effect of acetic acid (A), lactic acid (B), citric acid (C) and ethanol (D) on lactic acid production by the six selected LAB strains.

plantarum tested strains maintained their ability to produce lactic acid in proportions ranging from 35 to 66,96% of the initial level with a maximum of lactic acid (between 1.12 and 1.35%) produced under 0.5% of citric acid in the medium. The effect of acids results in a reduction of cytoplasmic pH that is likely to impair lactic acid production (Lopez and Dimick, 1995). This indicates that, compounds present at variable concentrations in the cocoa pulp such acids may be limiting factors for lactic acid production in LAB strains during cocoa fermentation.

However, with their ability to maintain lactic acid production despite pH fluctuations between 3 and 6 in cocoa beans fermentation, all the six selected LAB strains should be particularly interesting as starters.

Effect of ethanol on lactic acid production

The influence of ethanol concentration on lactic acid production is shown in Figure 2D. The results suggested

that, all these six studied strains maintained a good level of lactic acid production until 8% of ethanol concentration. This lactic acid production ranged between 43.50 and 91.67% of their initial potential production. These results are similar to those described by Behannis et al. (2012) with a decrease in acid produced when ethanol concentration increased from 2.5 to 7.5%. These results are interesting because ethanol is a major metabolite of cocoa pulp fermentation and the maximum rate of ethanol produced by yeasts is around 8% during this process (Lefeber et al., 2012). High concentrations of ethanol reduce cellular vitality and increase cell death resulting in a gradual decline in lactic acid production (Birch et al., 2000). Thus, these six ethanol tolerant isolates (T₁Gb₈, T₆C₅, T₆C₁₂, T₇C₈, T₉C₉ and T₁₁C₅) at high ethanol concentration, could both maintain their citrate and acetoin metabolism and their lactic acid production in the required standards to improve the final fermenting cocoa bean quality.

Conclusion

In this study, six LAB strains (T₁Gb₈, T₆C₅, T₆C₁₂, T₇C₈, T₉C₉ and T₁₁C₅) involved in Ivorian cocoa fermentation were identified as *Lactobacillus plantarum*. These particular cocoa-specific acidifying bacteria presented both the required acidifying capacity and in a lesser extent, citrate degradation and acetoin production. All of them were able to maintain their lactic acid production under fermentative stress conditions in the required standards. That make them suit candidate as starter for cocoa fermentation control.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

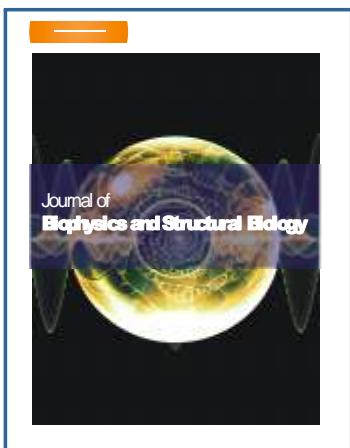
ACKNOWLEDGEMENTS

This work was supported by a Ph.D. grant to the first author. The authors are grateful to the National Flowers Center of Felix Houphouët-Boigny University for their availability and support during the fermentations.

REFERENCES

- Afoakwa OE, Kongor JE, Takrama JF, Budu AS (2013). Changes in acidification, sugars and mineral composition of cocoa pulp during fermentation of pulp preconditioned cocoa (*Theobroma cacao*) beans. *International Food Research Journal*, 20:1215-1222.
- Behannis M, Kayanush AJ (2012). Influence of Ethanol on Probiotic and Culture Bacteria *Lactobacillus bulgaricus* and *Streptococcus thermophilus* within a Therapeutic Product. *Journal of Medical Microbiology*, 2:70-76.
- Birch RM, Walker GM (2000). Influence of magnesium ions on heat shock and ethanol stress responses of *saccharomyces cerevisiae*. *Enzyme Biochemical Technology*, 26:678-687.
- Bonvehi JS, Coll FV (1997). Parameters affecting the quality of processed cocoa powder: acidity fraction. *Zeitschrift für Lebensmittel-Untersuchung und -Forschung*, 204:287-292.
- Crafack M, Mikkelsen MB, Saerens S, Krudsen M, Blennow A, Lowor S, Nielsen DS (2013). Influencing cocoa flavour using *Pichia kluyveri* and *kluyveromyces marxianus* in a defined mixed starter culture for cocoa fermentation. *International Journal of Food Microbiology*, 167:103-116.
- Deheuvelds O, Assiri AA, Petithuguenin P, Kebe BI, Flori A (2003). Production cacaoyere en cote d'ivoire : etat actuel du verger et pratiques paysannes –in: 14th International Cocoa Research Conference, Working paper, Accra (Ghana).
- Guha A, Soumitra B, Debabrata B (2013). Production of lactic acid from sweet meat industry waste by *lactobacillus delbruki*. *International Journal of Research in Engineering and Technology*, 2:630-634.
- ICCO 2016. International Cocoa Organization. www.icco.org Visited 06 juin 2016.
- Joko S, Ho E, Cahyaningsih, Betty SL Jenny (2014). Application of lactic acid bacteria to control microbial contaminants during fermentation of cocoa beans. *International Journal of Research and Agricultural in Food Science*, 2:16-24.
- Kempler GM, Kay Mc LL (1980). Improved medium for detection of citrate-fermenting *streptococcus lactis subsp. Diacetylactis*. *Journal of Applied and Environmental Microbiology*, 39: 927-956.
- King N (1948). Modification of Voges-Proskauer test for rapid colorimetric determination of acetyl methyl carbimol plus diacetyl in butter. *Dairy Industries*, 13:860-866.
- Kouame LM, Doue GG, Adom NJ, Ouattara HG, Niamke SL (2015). Biochemical characterization of microbial populations involved in Loh-Djiboua cocoa's fermentation in Cote d'Ivoire. *Food Environmental Safety*, 14:196-205.
- Lefeber T, Papalexandratou Z, Gobert W, Camu N, De Vuyst L (2012). On-farm implementation of starter culture for improved cocoa bean fermentation and its influence on the flavour of chocolate produced thereof. *Food of Microbiology*, 30:379-392.
- Lopez AS, Dimick PS (1995). Cocoa fermentation. In: Reed, G., Nagodawithana, T. W. (Eds.), *Enzymes, Biomass, Food and Feed*. VCH, Weinheim. pp 561-577.
- Ouattara HG, Reverchon S, Niamke SL, Nasser W (2011). Molecular identification and pectate lyase production by *Bacillus* strains involved in cocoa fermentation. *Food Microbiology*, 28:1-8.
- Papalexandratou Z, Lefeber T, Bahrin Lee SO, Daniel HM, De Vuyst L (2013). *Hanseniaspora Opuntiae*, *Saccharomyces Cerevisiae*, *Lactobacillus fermentum*, and *Acetobacter pasteurianus* predominate during well-performed Malaysian cocoa bean box fermentation, underlining the importance of these microbial species for a successful cocoa bean fermentation process. *Food Microbiology*, 35:73-85.
- Pereira GVD, Miguel M, Ramos CL, Schwan RF (2012). Microbiological and physicochemical characterization of small scale cocoa fermentations and screening of yeast and bacterial strains to develop a defined starter culture. *Applied and Environmental Microbiology*, 78:5395-5405.
- Raimbault M (1995). Importance des bactéries lactiques dans les fermentations du manioc. In *Transformation Alimentaire du Manioc*. T. Agbor Egbe, A. Brauman, D. Griffon, S. Trèche (éd ORSTOM).
- Rodriguez-Campos J, Escalona-Buendia HB, Contreras-Ramos SM, Orozco-Avila I, Jaramillo-Flores ME, Lugo-Cervantes (2012). Effects of fermentation time and drying temperature on volatile compounds in cocoa. *Food Chemistry*, 132:277-288.
- Serra BJ, Ventura CF (1997). Evaluation of bitterness and astringency of polyphenolic compounds in cocoa powder. *Food Chemistry*, 60:365-370.
- Schwan RF, Wheals AE (2004). The microbiology of cocoa fermentation and its role in chocolate quality. *Critical Reviews in Food Science and Nutrition*, 44:205-221.
- Yao A (2009). La fermentation du manioc en gari dans l'Afrique de l'Ouest : production d'un starter de bactéries lactiques lyophilisées. Thèse de Doctorat Unique, Université de Liège, 213 p.

Related Journals:



Related Journals:

